4-16-2013

Characterization of the Innate Immune Response in Sea Lamprey and Its Suppression in Ictalurid Catfish

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CHARACTERIZATION OF THE INNATE IMMUNE RESPONSE IN SEA LAMPREY AND ITS SUPPRESSION IN ICTALURID CATFISH

by

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A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

Major: Biology

The University of Memphis

May 2013

DEDICATION

This dissertation is dedicated to my mom, Becky Rose, my dad, David Rose, my sister, Sue Ann Bullard, my nephew, Brett Michael Bullard, and my partner in life, Amy Beth Hines. Without your love and support, I never would have made it. Thank you to all of my friends and family for bearing with me through the good and the bad.

ACKNOWLEDGMENTS

I am thankful to my mentor and advisor, Dr. Donald D. Ourth, for his guidance, direction, and, most of all, his unending patience. I am also grateful to my committee members, Dr. Chung, Dr. Wong, Dr. Freeman, and Dr. Simco for their input and support. I thank Dr. Beck for his sage advice and Dr. Biggers for all of the free pizza. I thank Dr. Taller and Dr. Cole for their assistance and the use of their lab equipment. I would like to thank Donna Haskins for her hard work, friendship and pep talks. A very special thank you to James Moore for getting me over that last hurdle.

I would also like to acknowledge my fellow graduate students, especially Manali Joglekar, Miriah S. Mertell, Deepthi Raghu and Gayatri Mammidanna. Thanks for being there when I needed you.

ABSTRACT

Rose, Wendy Michelle, PhD. The University of Memphis. May 2013. Characterization of the Innate Immune Response in Sea Lamprey and Its Suppression in Ictalurid Catfish. Major Professor: Donald D. Ourth.

The sea lamprey (*Petromyzon marinus*) belongs to the most primitive class of fish and has only innate immunity. Mannose-binding C-type lectin (MBL) was initially isolated by mannan-agarose affinity chromatography from sea lamprey plasma. The affinity-purified and 2-ME reduced lamprey MBL showed two bands of 35 kDa and 65 kDa by SDS-PAGE and Western blotting using guinea pig anti-MBL IgG as the primary antibody. N-terminal amino acid sequencing by Edman degradation for the first 10 residues gave XXXTKGCPDA. Lamprey plasma contained 261 μg of MBL/mL of plasma. Plasma protein concentration was 40.1 mg/mL. Lamprey MBL was present then in plasma at 6.5 μg MBL/mg total protein. The sea lamprey MBL specifically binds to mannose on the surface of the pathogen *Aeromonas salmonicida*. Plasma concentration of lamprey lysozyme was 5 μg lysozyme/mg total protein. Lysozyme and an antifungal peptide were isolated by low molecular weight gel filtration chromatography from sea lamprey plasma. Lysozyme and antifungal activity for each fraction were determined by well diffusion assay using Gram-negative bacteria, Gram-positive bacteria and two fungal species. The molecular weight of lamprey lysozyme was 14.3 kDa. The sea lamprey lysozyme was effective against Gram-positive bacteria but not against Gram-negative bacteria or fungi. Molecular weight of the antifungal peptide was approximately 3,000 Daltons. Antifungal plasma activity was seen against *Penicillium notatum* and *Aspergillus flavus*.

Decreased immunity from stress can lead to increased susceptibility of fish to infectious diseases. Thirty catfish were intraperitoneally injected with prednisolone acetate (PA) to simulate a long-term stress response. Blood samples were obtained from each catfish before and for five weeks after PA injection for a total of six weeks. Sera were assayed for total protein, albumin, globulin, albumin/globulin ratios (A/G), glucose, lysozyme and mannose-binding lectin (MBL). Total protein concentrations decreased 50% by week six in the PA-injected catfish when compared with pre-bled sera. Lysozyme and MBL levels all increased the week following PA injection and then decreased 40-50% by week six in the stressed catfish. Catfish with the highest pre-bled total serum protein concentrations survived the longest. Chemical-induced stress affected protein synthesis by significantly decreasing both serum and innate protein concentrations.

PREFACE

My main research objective was to isolate and characterize proteins involved in the innate immune response in fish. Fish are of interest and importance due to the divergence of adaptive immunity within the fish Class Chondrichthyes. This would indicate fish belonging to the most primitive Superclass of fish, Agnatha, may only have innate immunity. Fish belonging to the most advanced class of fish, Osteichthyes, have both innate and adaptive immunity. Both the Introduction and Conclusion are formatted in the style for publication in *Veterinary Immunology and Immunopathology*.

The research began with the sea lamprey (*Petromyzon marinus*) which belongs to the most primitive class of fish (Agnatha). The first objective was to determine if the innate immune protein, mannose-binding lectin (MBL), was present in the plasma of the sea lamprey and then isolate, characterize and determine its concentration for comparison with the bony fish concentration of MBL. These findings represent Chapter 2 of this dissertation, which was published in 2008 in the journal *Veterinary Immunology and Immunopathology*. MBL was also isolated, purified, and characterized in two different species of Ictalurid catfish using a primary antibody developed by Dr. Ourth (Ourth and Rose, 2011).

A second objective of the research was to isolate and characterize lysozyme from sea lamprey plasma and to determine if other antimicrobial peptides were present. An antifungal peptide was isolated and its concentration was determined. These results were published in *Veterinary Immunology and Immunopathology* in 2009 and make up Chapter 3 of this dissertation.

A third objective was to determine the effects of chemically-induced stress on innate immune proteins and other factors in Ictalurid teleost fish. We chose Ictalurid catfish because of their availability and economic importance in the aquaculture industry. At the University of Memphis, we have an aquaculture facility available for doing this type of suppression experiment. The results of this research are presented in Chapter 4 of this dissertation. This chapter has been formatted for publication in the journal, *Veterinary Immunology and Immunopathology*.

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Chapter 1: Introduction

Immunity is an intricate system of both innate and acquired defense mechanisms by an animal. Innate, or natural, immunity is the first line of defense against microbial invaders, responding within the initial stages of an infection (Pinchuk, 2002). Superclass Agnatha contains the jawless fish and are the most primitive vertebrates. The jawless Agnathans and all invertebrates are reliant on the innate immune response as these animals lack an adaptive immune response. Agnathans do not have recombination activating genes (RAG), expressed in lymphocytes, which provide the enormous variability and diversity found in the adaptive immune response (Guo et al., 2009). The RAG genes first appeared in the jawed fishes. The innate immune system has become an important defense mechanism for studying fish immunity, as fish are the first vertebrates and therefore may rely more on innate immunity than adaptive immunity (Magnadottir, 2006; Whyte, 2007). The fish immune system is considered a crossroads between the innate and adaptive immune responses (Tort et al., 2003). This means that fish may be even more dependent on innate immunity than other vertebrates.

Adaptive immunity is thought to have co-evolved with the first jawed vertebrates, the cartilaginous fishes, approximately 600 million years ago (Nonaka, 2006). All animals from cartilaginous fish to mammals possess both innate and adaptive immune responses. Mediators of the adaptive immune response are T-cells, B-cells, antibodies, and major histocompatibility complex proteins (Suckale, 2005). Adaptive immunity is considered adaptive or specific, not only because it can distinguish self from non-self (as does innate immunity), but it also confers protection against re-infection or a memory response to the same microbe (Fujita, 2004). Thus the adaptive immune response has memory to pathogens and mounts a faster, more specific response with each successive

encounter with the same pathogen. The appearance of adaptive immunity did not render the innate immune response obsolete. It is the innate immune system that first recognizes the microbial invader using pattern-recognition receptors for sensing an infection and leading to an adaptive immune response (Holland, 2001).

Innate immunity is the natural immunity conferred to an organism at birth. It includes barriers such as skin, mucus membrane surfaces, and chemical substances with antimicrobial capabilities. Lysozyme, proteins that neutralize microorganisms through complement activation like mannose-binding lectin (MBL), and cells that specialize in phagocytosis are included here. These responses are considered non-adaptive or non-specific because they do not change no matter what the infection is or the number of times this infection is encountered (Pinchuk, 2002). The majority of multicellular animals in existence (approximately 98.6%) have only innate immunity, indicating that an adaptive immune response is not necessary for their survival (Watts, 2001).

Of the components of the innate immune response, the proteins MBL and lysozyme are of prime importance. Based on research by Gajeva et al. (2004), it is known that low serum levels of MBL are directly related to disease susceptibility in humans. Lectins are proteins that recognize specific carbohydrates on the surfaces of bacteria, viruses and fungi that are not present on the surfaces of animal cells (Jack, 2003). Soluble lectins located in mammalian plasma play an important role in the innate immune response and are the first line of defense in recognizing pathogens as non-self. Once the carbohydrate moiety or mannose of a pathogen is recognized, MBL binds or opsonizes the non-self pathogen marking it for phagocytosis by macrophages (Hoffman, 1999). During an infection or in response to an inflammatory response, MBL levels

increase and act as opsonins to activate the mannan-binding lectin complement pathway. This leads to an innate immune response and death of the pathogen (Turner, 2003; Fujita, 2002).

MBL has not before been identified and quantitated in the plasma of the Agnathan sea lamprey species, *Petromyzon marinus*. In Chapter 2, Mannose-binding C-type lectin was purified from sea lamprey plasma by mannan-agarose affinity chromatography and quantitated. The lamprey MBL was then characterized by SDS-PAGE and Western blotting using guinea pig IgG to MBL, N-terminal amino acid sequencing and amino acid composition analysis. Binding experiments were done that demonstrated the specific binding of sea lamprey biotinylated MBL to mannose on the surface of the pathogen *Aeromonas salmonicida*. The isolation of MBL by affinity chromatography is evidence for a mannan-binding lectin complement pathway in sea lamprey plasma. The presence of MBL in plasma of this sea lamprey species could be important in their innate immunity and disease resistance. The sea lamprey species investigated here migrated from the Atlantic Ocean into the North American Great Lakes in the 19th century. This lamprey species is a parasitic cartilaginous fish of the North American Great Lakes and a predator of many fish species of commercial importance to the fishing industry.

Lysozyme is an important protein of the innate immune response and is found in both invertebrates and vertebrates. The enzymatic action of lysozyme is directed against the peptidoglycan cell wall of bacteria resulting in cell wall lysis. Lysozyme has been found in the plasma, mucus, and ova of many teleost fish species (Ourth, 1980; Ourth and Wilson, 1981; Lie et al., 1989; Yousif et al., 1994; Ellis, 1999). In mammals and jawed fish, lysozyme lyses the cell walls of Gram-positive bacteria and is a major component in

resistance to bacterial infections (Grinde, 1988). Teleost bony fish lysozyme has been shown to be effective against both Gram-positive and Gram-negative bacteria (Jolles and Jolles, 1984; Grinde, 1989). Lysozyme is an important indicator of the innate immune response in fish (Saurabh and Sahoo, 2008).

Antifungal peptides are also important factors of innate immunity (DeLucca and Walsh, 1999, 2000). These peptides have been isolated from bacteria, fungi, plants and animals including fish. Crude extracts from the freshwater fish *Channa striatus* were found to possess antifungal activity against several fungal species (Mat Jais et al., 2008). Antifungal peptides exert their action through lysis by disrupting outer membranes leading to pore formation (DeLucca and Walsh, 1999).

In Chapter 3, *P. marinus* plasma was tested by well diffusion assay against Gram-negative bacteria, Gram-positive bacteria and fungi to determine the presence of lysozyme and other antimicrobial peptides. The positive results for lysozyme and antifungal activity in plasma led to the isolation of these two innate immune factors by gel filtration column chromatography. Further characterization of lysozyme and antifungal peptide was done by gel fraction well diffusion assay, SDS polyacrylamide agarose gel electrophoresis (SDS-PAGE) and low molecular weight standards. Chapter 3 is the first time lysozyme and antifungal activity have been identified, isolated, and characterized in plasma of this sea lamprey species.

Catfish belong to the Class Osteichthyes, Infraclass Teleostei (bony fish). All bony fish have both innate and adaptive immunity and are thus considered to be the most advanced fish. The channel catfish is extensively used in aquaculture in the Southeastern USA. Catfish can be subjected to stressful pond conditions which contribute to fish

morbidity and mortality (Small and Bilodeau, 2005). Stress can be physical, such as overcrowding, poor water quality and handling, or chemical toxicants (Woo and Bruno, 2009). The onset of stress leads to an increase in circulating plasma cortisol and glucose or the fight-or-flight response (Small and Bilodeau, 2005). Cortisol is a glucocorticoid and is the primary component of the stress response in fish. In teleost fish, cortisol is also an important factor in metabolism, growth and osmoregulation (Mommsen et al., 1999)

Studies have been done correlating the effects of acute stress on the immune response of catfish (Ellsaesser and Clem, 1987; Bilodeau et al., 2005; Small and Bilodeau, 2005). These studies show that short-term or acute stress may even be beneficial to the immune health of farmed fish, initially stimulating both innate and adaptive immune responses for increased disease resistance (Maule et al., 1989; Demers and Bayne, 1997; Bilodeau et al., 2003; Bilodeau et al., 2005). With acute or short-term stress, the increase in cortisol and glucose is only temporary lasting no longer than 24 hours. Long-term elevation in plasma cortisol associated with chronic stress can lead to immunosuppression and higher rates of infection in fish (Ellsaesser and Clem, 1987; Bilodeau et al., 2005).

In Chapter 4, the stress response was chemically-induced by injecting catfish with prednisolone acetate which mimics cortisol (McCarthy, 1977). Prednisolone acetate (PA) has been used in other studies to induce a stress response and suppress immunity to reveal latent infections (Hiney et al., 1994; Cipriano et al., 1997). However, the effects of chemical suppression on serum protein levels and two proteins of the innate immune response, lysozyme and MBL, have not before been reported until now in this Ictalurid hybrid aquaculture species.

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Chapter 2: Isolation of mannose-binding C-type lectin from sea lamprey (*Petromyzon marinus*) plasma and binding to *Aeromona salmonicida*

1. Introduction

The sea lamprey species investigated here migrated from the Atlantic Ocean into the North American Great Lakes in the 19th century. This lamprey species is a parasitic cartilaginous fish of the Great Lakes region and a predator of many bony fish species of commercial importance to the fishing industry.

Lectins are proteins that recognize specific carbohydrates. Mammalian lectins play an important role in innate immunity and disease resistance (Fujita, 2002; Jack and Turner, 2003; Turner, 2003; Gadjeva et al., 2004). Soluble plasma lectins are a first-line of host defense that can initially recognize pathogens as non-self. This carbohydrate recognition leads to phagocytosis by macrophages and enhances complement-mediated cell lysis. Collectins are soluble lectins found in mammals and birds (Vitved et al., 2000) and are C-type (calcium-dependent) lectins composed of multiple subunits (Fujita, 2002; Jack and Turner, 2003; Turner, 2003; Gadjeva et al., 2004). The carbohydrate-recognition domains of collectins recognize specific carbohydrate patterns (which includes mannose) on the surfaces of bacteria, viruses and fungi that are not present as surface carbohydrates of higher eukaryotes, and so help to distinguish self from non-self (Fujita, 2002; Jack and Turner, 2003; Turner, 2003; Gadjeva et al., 2004). The collectins opsonize non-self microorganisms and also activate the lectin complement pathway, leading to an innate immune response and the killing of Gram-negative bacteria and enveloped viruses (Hoffmann et al., 1999; Suckale et al., 2005; Kindt et al., 2007). Mannose-binding lectin therefore acts as a first-line of defense against many microbial pathogens. Mannose-binding lectins have been reported in trout, salmon, carp, rohu fish,

channel catfish, blue catfish (Jensen et al., 1997; Ewart et al., 1999; Vitved et al., 2000; Mitra and Das, 2002; Ourth et al, 2007) and now here in plasma of the sea lamprey species, *Petromyzon marinus.*

Mannose-binding C-type lectin (MBL) is an important component of innate immunity in mammals (Fujita, 2002; Jack and Turner, 2003; Turner, 2003; Gadjeva et al., 2004). Mannose-binding lectin, an acute-phase protein produced by hepatocytes, increases in response to an infection or inflammatory response. The innate immune system has become important for studying fish immunity (Magnadottir, 2006; Whyte, 2007). Mannose-binding lectin has not before been identified and quantitated in the plasma of the sea lamprey species, *P. marinus*.

Mannose-binding C-type lectin was initially purified from sea lamprey plasma by mannan-agarose affinity chromatography and quantitated. The lamprey MBL was then characterized by SDS-PAGE and Western blotting using guinea pig IgG to MBL, N-terminal amino acid sequencing and amino acid composition analysis. Binding experiments were done that demonstrated the specific binding of sea lamprey biotinylated MBL to mannose on the surface of the pathogen *Aeromonas salmonicida*. The isolation of MBL by affinity chromatography is evidence for a mannan-binding lectin complement pathway in sea lamprey plasma. The presence of MBL in plasma of this sea lamprey species could be important in their innate immunity and disease resistance.

2. Materials and Methods

2.1. Sea Lamprey Plasma

Lamprey blood was obtained by cardiac puncture from eight adult lamprey of unknown sex on their spawning run. The blood was collected using the anticoagulant heparin. Following centrifugation at 400 xg for 15 min., the 30 ml pool of lamprey plasma obtained was stored at -80^{o}C.

2.2. Production of Guinea Pig IgG Antibody to Mannose-Binding Lectin

Two ml of rabbit mannose-binding lectin bound to agarose gel (Pierce Chemical Co., Rockford, IL) were emulsified in 2 ml of Freund's complete adjuvant (Benstein and Ourth, 2004; Ourth et al., 2005; Ourth et al., 2007). Four guinea pigs were each initially immunized subcutaneously (SC) with 1 ml of the emulsion. Four months later, each guinea pig was again immunized SC with 1 ml of the same vaccine, but this time containing Freund's incomplete adjuvant. Two months later, the guinea pigs were bled for serum antibody to MBL.

A Protein A-agarose affinity column (Sigma Chemical Co., St. Louis, MO) was used to isolate guinea pig IgG to MBL according to their procedure. The IgG was eluted with 0.2 M Na_2HP0_4/0.1 M citric acid, pH 3.5. The IgG antibody to MBL was then used as the primary antibody in Western blot analysis to identify lamprey MBL.

2.3. Purification of Lamprey Mannose-Binding Lectin by Affinity Chromatography

The following buffers adapted from Nevens et al. (1992) were prepared for affinity chromatography: Mixing buffer (20 mM imidazole-HCl, pH 7.8, 2.5 M NaCl, 40 mM $CaCl_2$); Loading buffer (10 mM imidazole-HCl, pH 7.8, 1.25 M NaCl, 20 mM $CaCl_2$); Elution buffer (10 mM imidazole-HCl, pH 7.8, 1.25 M NaCl, 3 mM EDTA).

Sea lamprey plasma was centrifuged at 27,000 xg for 15 min. at 4^{o} C. Twenty-five ml of lamprey plasma (40.1 mg/ml protein; representing a plasma pool of 8 lamprey) were mixed with 25 ml of mixing buffer and stirred for 60 min. at 24^{o}C. This mixture was applied to a mannan-agarose affinity column (Sigma, St. Louis, MO), and the peak was then eluted with 10 ml of elution buffer containing 3 mM EDTA (Fig. 2.1). The EDTA chelates calcium thus releasing MBL from the affinity column. Two ml per tube fractions were collected and monitored at 280 nm. The affinity chromatography isolation procedure according to Nevens et al. (1992) was followed.

2.4. SDS-Polyacrylamide Gel Electrophoresis and Western Blotting of Lamprey Mannose-Binding Lectin

SDS-PAGE (12% separating gel; 4% stacking gel) was performed under reducing (with 2-ME) and non-reducing conditions (Laemmli, 1970; Kawasaki et al., 1989; Chung and Ourth, 2000; Ourth et al., 2005; Ourth et al., 2007) to identify the affinity-purified lamprey MBL (Fig. 2.2). 7.2 μg of the purified and 2-ME reduced lamprey MBL was applied to lane 1 (Fig. 2.2). The gel (8 x 10 cm and 0.75 mm thick) was subjected to electrophoresis at 20 mA for 60 min. Molecular weight (75 kDa, 50 kDa, 37 kDa, 25 kDa) protein standards (Precision Plus, Bio-Rad, Hercules, CA) were used.

Western blot analysis was done several times by electrophoretic transfer to nitrocellulose membrane to identify the lamprey MBL using guinea pig IgG antibody to MBL as the primary antibody (Ourth et al., 2007). The transfer voltage was 12 v done for 90 min. on ice using a 25 mM Tris-Glycine/20% methanol, pH 8.3 buffer. The nitrocellulose membrane was removed from the blotting apparatus and put in a dish. Non-specific protein sites were blocked with 1% purified casein solution in Tris buffered

saline, pH 7.5 (TBS) for 2 hr. After washing the membrane three times with TBS, 20 ml of a 1:200 dilution in TBS of the guinea pig antirabbit-MBL IgG was added and incubated for 4 hr at room temp. as the primary antibody. After washing the membrane again three times in TBS, a 1:500 dilution in TBS of rabbit antiguinea pig IgG-horseradish peroxidase conjugate (Sigma, St. Louis, MO) was added and incubated for 60 min. at room temp. as the secondary antibody. This was followed by washing the membrane three times in TBS. A 3,3'-diaminobenzidine tetrahydrochloride solution was then used to develop the brown colored product for 15 min. and the reaction stopped with water.

2.5. N-terminal Amino Acid Sequencing and Amino Acid Composition Analysis of Lamprey Mannose-Binding Lectin

N-terminal amino acid sequencing (Edman degradation) and amino acid composition analysis of the affinity-purified lamprey MBL were determined. This was done by Dr. Brian Morrison at the Molecular Analysis Facility, University of Iowa College of Medicine, Iowa City, IA.

2.6. Binding of Lamprey Mannose-Binding Lectin to Aeromonas salmonicida

The sea lamprey MBL was biotinylated according to instructions provided by Pierce Chemical Co., Rockford, IL. Fifty µl (33 µg) of biotinylated lamprey MBL were incubated with 100 µl of a 1:10 dilution of a 48 hr culture of *Aeromonas salmonicida* for 1 hr at 24 ^{0}C. This was followed by washing four times in phosphate-buffered saline, pH 7.2 (PBS) with centrifugation for 5 min. at 11,000 xg. The fourth wash was used as a control in the dot-blot ELISA to monitor the binding of the lamprey MBL to the bacteria. The pellet was then reconstituted to 50 µl with 200 mM mannose to displace the bound

MBL from the bacteria. The mixture was then incubated for 30 min. at 24 ^{0}C with intermittent vortexing and then spotted onto nitrocellulose membrane for the dot-blot ELISA technique. This was followed by incubation of the membrane for 1 hr at 24 ^{0}C in 1% casein and then washed three times in PBS. The membrane was treated with a 1:500 dilution of streptavidin-HRP (Sigma, St. Louis, MO) for 1 hr at 24 ^{0}C and washed again three times in PBS. A 3, 3'-diaminobenzidine tetrahydrochloride (DAB) solution (10 mg DAB, 10 μl 30% H_2O_2, 15 ml PBS) was added for color development. Color development was for 5 min. and the reaction stopped with water.

2.7. Protein Assay

Protein concentrations of the affinity-purified lamprey MBL and lamprey plasma were determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was used as the protein standard.

3. Results and Discussion

A mannan-agarose affinity column was used to purify the sea lamprey MBL (Fig. 2.1). Mannan was immobilized on cross-linked 4% agarose beads (Sigma, St. Louis, MO). A single peak containing lamprey MBL was eluted from the mannan-agarose affinity column (Fig. 2.1) using an EDTA-containing elution buffer that chelates calcium and releases MBL (Kawasaki et al., 1989; Nevens et al., 1992; Ourth et al., 2005; Ourth et al., 2007).

Figure 2.1: Isolation of Mannose-Binding C-type Lectin from Sea Lamprey Plasma

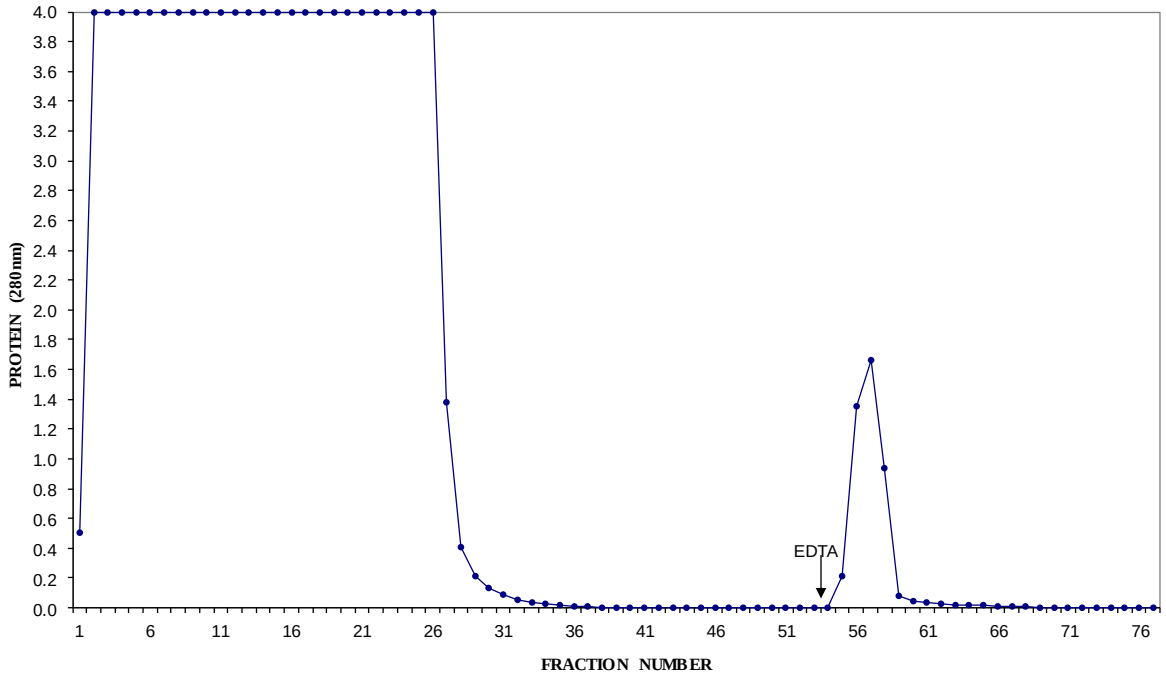


Figure 2.1 Isolation of mannose-binding C-type lectin from sea lamprey plasma by affinity chromatography. Lamprey plasma was applied to a mannan-agarose affinity column (Sigma) and eluted with 3 mM EDTA. Two mL per tube fractions were collected.

SDS-PAGE (12% gel) and Western blotting of the 2-ME reduced lamprey MBL showed two molecular weight bands of 35 kDa and 65 kDa with the primary IgG antibody (Fig. 2.2). This could indicate the presence of two different isoforms or subunits of the sea lamprey MBL. No band was seen to enter the gel with the unreduced affinity-purified lamprey MBL by SDS-PAGE and Western blotting, indicating a molecular mass of large molecular weight. The primary IgG antibody produced was specific for human and rabbit MBL (Benstein and Ourth, 2004), channel catfish MBL and blue catfish MBL (Ourth et al., 2007) and was also specific here for lamprey MBL by Western blot analysis (Fig. 2.2).

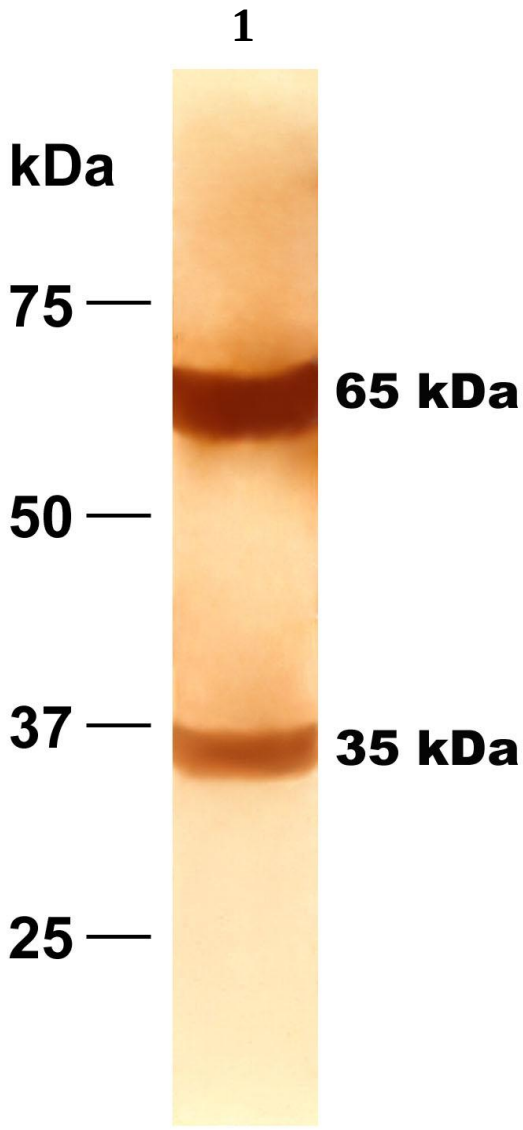


Figure 2.2. SDS-PAGE-Western Blot analysis of purified mannose-binding lectin isolated by mannan-agarose affinity chromatography from sea lamprey plasma

Mannose-binding C-type lectins, previously isolated from channel catfish and blue catfish sera, had a molecular weight range of 62 kDa- 66 kDa (Ourth et al., 2007). The 65 kDa lamprey C-type lectin isolated here (Figs. 2.1 and 2.2) agrees then with this previous finding for catfish. Schluter et al. (1994) also found two subunits by SDS-PAGE of 35 kDa and 65 kDa in a C-type lectin isolated from sea lamprey (*Petromyzon marinus*) fish eggs. No quantitation of the egg lectin nor identification of the lectin in lamprey plasma were done.

The mixing and loading buffers used both contained calcium necessary for binding MBL to the mannan-affinity column. The C-type lectin binds mannose in a calcium-dependent manner as the lamprey lectin could subsequently be eluted from the mannan-agarose affinity column with 3 mM EDTA (Fig. 2.1). EDTA is a chelator of calcium. The lamprey MBL can be considered a C-type lectin (calcium-dependent) since it could be eluted from the mannan-agarose affinity column with EDTA. Lamprey MBL is likely then a member of the collectin family of calcium-dependent proteins. In mammals and birds, MBL is a collectin composed of subunits containing three identical polypeptide chains of about 30 kDa (Vitved et al., 2000). The lamprey C-type lectin purified in this study is composed of 35 kDa and 65 kDa polypeptides and may represent a different lectin subfamily.

Only one N-terminal end amino acid sequence was detected for the isolated lamprey MBL, which demonstrated purity of the affinity-purified MBL. No N-terminal blockage was found. This indicates that the N-terminal end amino acid sequence is the same for both molecular weight bands, but that the C-terminal end of the 35 kDa segment likely represents post-translational processing. N-terminal amino acid sequencing by

Edman degradation of the sea lamprey plasma MBL for the first 10 residues gave XXXTKGCPDA, where X represents an unidentified amino acid. A database Blast Search found a sequence match of the sea lamprey plasma 4-10 amino acid sequence isolated here with a 35 kDa subunit of C-type lectin isolated from sea lamprey (*Petromyzon marinus*) fish eggs for which a 16 amino acid sequence was obtained (Schluter et al., 1994). They did not determine if this C-type lectin was also found in sea lamprey plasma.

The amino acid composition analysis of the affinity-purified lamprey plasma MBL found differences when compared with the three other vertebrate serum MBLs (Table 2.1). The amount of histidine present (mol%) in lamprey MBL was over 2-times greater when compared with the amount of histidine present in Atlantic salmon and human MBLs. The amount of threonine present (mol%) in lamprey MBL was 2-times greater when compared with Atlantic salmon MBL. The amount of tyrosine present (mol%) in lamprey MBL was over 3-times greater when compared with human MBL. The amount of phenylalanine present (mol%) was 3-times greater when compared with chicken MBL. Cysteine and methionine were present as cysta and met-S. The presence of cysteine as cysta indicates the presence of disulfide bonds in the sea lamprey MBL.

Table 2.1

Amino acid compositions (mol%) of sea lamprey plasma mannose-binding lectin (MBL) compared with three other vertebrate serum mannose-binding lectins

Amino Acid	Sea Lamprey MBL	Salmon MBL*	Chicken MBL*	Human MBL*
Cysta	1.1			
Asx	11.8	11.3	11.8	8.6
Met-S	0.37			
Glx	10.4	14.2	10.1	13.6
Ser	8.7	5.5	6.5	8.3
Gly	11.9	16.6	9.7	14.8
His	2.6	1.1	1.9	1.2
Arg	3.6	5.1	4.1	3.2
Thr	6.6	3.4	3.7	7.2
Ala	7.9	8.3	6.4	7.9
Pro	4.4	5.4	5.8	5.4
Tyr	3.8	2.4	2.7	1.1
Val	5.8	6.1	4.5	4.5
Met	D	0.4	1.3	ND
Ile	4.1	2.4	3.9	3.0
Leu	6.6	5.4	8.8	7.6
Phe	5.2	5.4	1.8	4.3
Lys	5.3	5.0	8.2	6.7
Trp	D	D	D	D
1/2 Cys	D	2.1	2.8	2.6

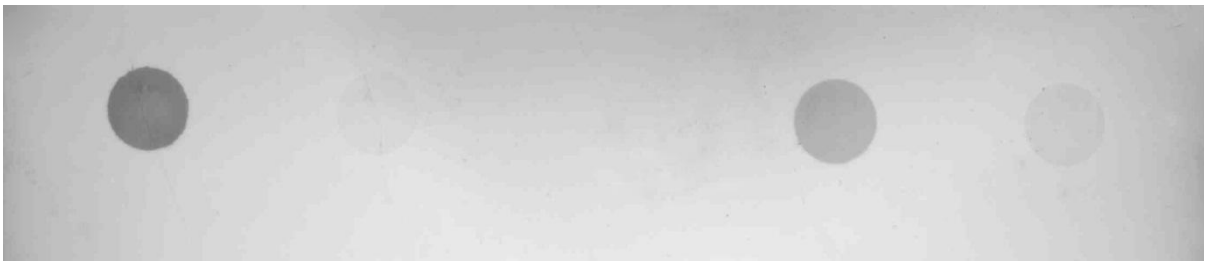
*Sources: Atlantic salmon mannose-binding lectin (Ewart et al. 1999); Chicken mannose-binding lectin (Laursen et al. 1995); Human mannose-binding lectin (Kawasaki et al. 1983). ND, none detected; D, destroyed by standard HCl hydrolysis

The total protein of the eluted peak (five tubes numbered 55-59) was 6.52 mg after purification by affinity chromatography (Fig. 2.1). From the amount of lamprey plasma (25 ml) used for initial purification by affinity chromatography, this was calculated to be 261 μg of MBL per ml of lamprey plasma. Lamprey plasma protein concentration was 40.1 mg/ml. Lamprey MBL was present then in lamprey plasma at 6.5 μg MBL/mg total protein. The presence of MBL in such high concentration in sea lamprey plasma could be important in innate immunity and their resistance to infection.

The sea lamprey plasma MBL isolated here (261 μg/ml) had 14.5-times the concentration of MBL in μg/ml when compared with the combined concentrations (18 μg/ml) of channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) serum MBLs (D. D. Ourth and W. M. Rose, University of Memphis, unpublished data). The sea lamprey plasma MBL must play an important role then in lamprey innate immunity to infectious diseases when compared with bony teleost fish.

The sea lamprey C-type lectin had specificity for mannose. Displacement of the isolated lamprey MBL from the *Aeromonas salmonicida* surface by mannose demonstrated that the lamprey lectin specifically binds mannose on the bacterial surface (Fig. 2.3). The specific binding of the sea lamprey biotinylated MBL to mannose on the surface of the pathogen *Aeromonas salmonicida* was demonstrated in Dot 1 using the dot-blot ELISA technique (Fig. 2.3). The lamprey MBL is able then to recognize mannose on the surface of this lamprey bacterial pathogen (Woo and Bruno, 1999). The biotinylated MBL control (Dot 4) was also positive by the dot-blot ELISA procedure demonstrating that the lamprey MBL had been successfully labeled with biotin (Fig. 2.3). There was very low binding of the streptavidin-HRP to *Aeromonas salmonicida* (Dot 5),

indicating that biotin was present in very low amount on the surface of this bacterial species (Fig. 2.3).



1 2 3 4 5

Figure 2.3: Binding of sea lamprey biotinylated MBL to *Aeromonas salmonicida* by the Dot-blot ELISA technique. The biotinylated C-type lectin specifically bound to *Aeromonas salmonicida* and was detected using streptavidin-HRP as described in the methods section. Dot 1: Biotinylated MBL was first bound to *Aeromonas salmonicida* and then displaced with 200 mM mannose; Dot 2: Fourth wash with phosphate-buffered saline of biotinylated MBL bound to *Aeromonas salmonicida* before displacement with 200 mM mannose; Dot 3: Phosphate-Buffered Saline; Dot 4: Biotinylated MBL only; Dot 5: *Aeromonas salmonicida* only

A lamprey serum N-acetylglucosamine (GlcNAc)-binding lectin was purified by affinity chromatography from *Lampetra japonicus* (Matsushita et al., 2004). The lamprey lectin consisted of 24 kDa subunits which formed a dimeric structural unit that could be an orthologue of mammalian C1q (Matsushita et al., 2004) and may be the lectin ficolin in which the acetyl group is needed for binding (Magnadottir, 2006; Whyte, 2007). Takahashi et al. (2006) purified lamprey (*Lampetra japonica*) MBL from serum, also using a GlcNAc-agarose affinity column, and found a major band at 25 kDa and a minor band at about 70 kDa with the reduced lamprey lectin. Ficolin and MBL both bind

GlcNAc (Janeway et al., 2005). MBL is also able to bind mannose-containing carbohydrates which the ficolins do not recognize (Janeway et al., 2005). The unreduced lamprey lectin was 300 kDa by SDS-PAGE (Takahashi et al., 2006). No N-terminal amino acid sequencing of this lamprey lectin was done. They also identified the presence of mannose-binding lectin associated serine protease (MASP) in this lamprey species. *Lampetra japonica* is an Arctic Ocean lamprey species.

A mannose-binding C-type lectin was purified from sea lamprey plasma by affinity chromatography (Figs. 2.1 and 2.2) and specifically bound to mannose on the surface of *Aeromonas salmonicida* (Fig. 2.3), a bacterial pathogen of the sea lamprey (Woo and Bruno, 1999). Our initial finding of MBL in the plasma of this sea lamprey species is important in understanding lamprey innate immunity and resistance to microbial pathogens. The presence of MBL in such high concentration in lamprey plasma (261 μg MBL/ml plasma) could be important in their innate resistance to bacterial infections, as the sea lamprey MBL bound to the pathogen *Aeromonas salmonicida* (Fig. 2.3). This study describes the presence of MBL in sea lamprey (*Petromyzon marinus*) plasma and evidence for a C-type lectin complement pathway of innate immunity.

Acknowledgement

This work was supported in part by Merck & Co., Inc., Rahway, NJ.

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Chapter 3: Isolation of lysozyme and an antifungal peptide from sea lamprey (*Petromyzon marinus*) plasma

1. Introduction

The sea lamprey species (*Petromyzon marinus*) investigated here is a parasitic cartilaginous fish of the class Agnatha. Agnathans are the most primitive fish lacking jaws and having no thymus or classical adaptive immune response (Mayer et al., 2002; Matsushita et al., 2004; Yun and Li, 2007; Raftos and Raison, 2008). This lamprey migrated from the Atlantic Ocean into the North American Great Lakes in the 19th century. *Petromyzon marinus* is a predator of many bony fish species of commercial importance to the Great Lakes fishing industry (Henderson, 1986; Li et al., 2003; Sorenson et al. 2005).

The innate immune system has received increasing attention as being of major importance in studying disease resistance in fish (Magnadottir, 2006; Whyte, 2007). Lysozyme is an important protein of the innate immune response and is found in both vertebrates and invertebrates. The enzymatic action of lysozyme is directed against the peptidoglycan cell wall of bacteria resulting in cell wall lysis. Lysozyme has been found in the plasma, mucus, and ova of many teleost fish species (Ourth, 1980; Ourth and Wilson, 1981; Lie et al., 1989; Yousif et al., 1994; Ellis, 1999). Teleost fish lysozyme has been shown to be effective against both Gram-positive and Gram-negative bacteria (Jolles and Jolles, 1984; Grinde, 1989).

Antifungal peptides are also important factors of innate immunity (DeLucca and Walsh, 1999, 2000). These peptides have been isolated from bacteria, fungi, plants and animals, including fish. Crude extracts from the freshwater fish *Channa striatus* were found to possess antifungal activity against several fungal species (Mat Jais et al., 2008).

Antifungal peptides exert their action through lysis by disrupting outer membranes leading to pore formation (DeLucca and Walsh, 1999).

Petromyzon marinus plasma was tested by well diffusion assay against Gram-negative bacteria, Gram-positive bacteria and fungi to determine the presence of lysozyme and other antimicrobial peptides. The positive results for lysozyme and antifungal activity in the plasma led to the isolation of these two innate immune factors by gel filtration column chromatography. Further characterization was done by gel fraction well diffusion assay, SDS polyacrylamide agarose gel electrophoresis (SDS-PAGE), and low molecular weight standards. This is the first time lysozyme and antifungal activity have been identified, isolated, and characterized in plasma of this sea lamprey species.

2. Materials and Methods

2.1. Sea Lamprey Plasma

Lamprey blood was obtained by cardiac puncture from eight healthy adult lamprey of unknown sex on their spawning run. The blood was collected using the anticoagulant heparin. Following centrifugation at 400 xg for 15 min, a 30 ml pool of lamprey plasma was obtained and stored at -80^{o}C.

2.2. Lamprey Plasma Well Diffusion Assay

A gel well diffusion assay in tryptic soy agar (TSA) was done to determine lysozyme activity of the lamprey plasma pool (Ourth, 1980; Ourth and Wilson, 1981; Lockey and Ourth, 1996; Chung and Ourth, 2000). Molten TSA (10 ml) was mixed with 60 µl of a 10^{-2} dilution of log growth phase (24 h culture) of *Micrococcus luteus* [ATCC 4698]. Ten µl of plasma were added to triplicate wells cut in the agar gel plate, and

plates were incubated for 48 h at 37^{o}C. Hen egg white lysozyme (Sigma, St. Louis, MO), done in duplicate wells, was used to construct a standard curve to quantitate (μg/ml) the lamprey lysozyme. Sterile saline was used as a negative control. A Kallestad (Chaska, MN) calibrated viewer was used to measure in mm the zones of growth inhibition around each well. The concentration of lamprey plasma lysozyme was determined using a standard curve of hen egg white lysozyme of known concentrations (200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml).

The sea lamprey plasma was tested by the same well diffusion assay to determine bactericidal activity against *Escherichia coli* [ATCC 14949]and *Aeromonas salmonicida* [ATCC 14174] (Chung and Ourth, 2000). Antifungal plasma activity against *P. notatum* and *A. flavus* was also determined by the well diffusion assay (Magaldi et al., 2004).

2.3. Purification of Lysozyme and Antifungal Peptide by P-10 Gel Filtration Column Chromatography

One ml of sea lamprey plasma was centrifuged at 3512 xg for 5 minutes. 0.5 ml (20 mg protein) of the supernatant was applied to a 1 cm x 50 cm column of Bio-Gel P-10 Polyacrylamide Gel (Bio-Rad, Hercules, CA). The column was equilibrated with 0.1 M NaH_2PO_4 buffer, pH 7.4, and lysozyme and antifungal peak fractions were eluted using this buffer. The elution flow rate was 1 ml/11 min and 1 ml fractions were collected. The fractions were assayed by spectrophotometry at 280 nm for protein absorbance and then tested for lysozyme activity and antifungal activity by the well diffusion assay (Chung and Ourth, 2000; Magaldi et al., 2004).

2.4. Well Diffusion Assay Using P-10 Column Chromatography Fractions

Cultures of *M. luteus*, *Bacillus megaterium* [ATCC 14581], *Mycobacterium phlei* [ATCC 354], *A. salmonicida*, and *E. coli* were prepared in tryptic soy broth and allowed to incubate at 37°C for 24 h. For each bacterium, 100 µl was added to 10 ml of saline and vortexed. 100 µl of this suspension was pipetted into 10 ml of molten TSA, poured into a petri dish, and allowed to solidify. Once the TSA solidified, 3 mm wells were punched and the agar plugs then removed by sterile pipette. Ten µl of each fraction collected from the P-10 column were pipetted into the wells. The agar plates were placed in an incubator at 37°C for 48 h. Zones of inhibition around each well were measured in mm using a Kallestad calibrated viewer after 48 h and were compared with wells containing 10 µl of hen egg white lysozyme (100 µg/ml).

Cultures of the fungal species, *A. flavus* and *P. notatum*, were inoculated on potato dextrose agar media. Three millimeter wells were punched in the agar and plugs removed. Twenty µl of P-10 column fractions were pipetted into each well. The plate was incubated at 25 °C for 24 h. 0.1 M NaH_2PO_4 buffer, pH 7.4, was used as a control well. Zones of inhibition were measured as previously described.

2.5. Molecular Weight Determination of Lysozyme

The molecular weight of the sea lamprey lysozyme was determined using SDS-PAGE (Laemmli, 1970). A 4-20% linear gradient pre-cast mini gel was used (Bio-Rad). 25 µl of hen egg white lysozyme (100 µg/ml) plus 5 µl of sample loading buffer were pipetted into lane 1. 25 µl of fraction #27 from P-10 column isolation plus 5 µl of sample loading buffer were pipetted into lane 2. 20 µl of Precision Plus (Bio-Rad, Hercules, CA) Protein Standards (75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 15 kDa, 10 kDa) were

pipetted into lane 3. Electrophoresis was carried out at 100 V and 20 mA for 90 min. The gel was stained for protein with Coomassie brilliant blue.

2.6. Molecular Weight Determination of Antifungal Peptide

Gel filtration column chromatography (Bio-Gel P-10) and low molecular weight standards were used to determine the molecular weight of the antifungal peptide. The low molecular weight standards used were hemocyanin (8,000 Daltons), insulin (5,800 Daltons), melittin (2,870 Daltons) and vitamin B-12 (1,350 Daltons).

2.7. Protein Assay

Protein concentrations were determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was used as the protein standard.

3. Results and Discussion

Sea lamprey plasma had a protein concentration of 40.1 mg protein/ml of plasma by BCA assay. The sea lamprey plasma pool had a lysozyme concentration of 5 μg lysozyme/mg total lamprey protein as determined by gel diffusion assay in comparison with total protein concentration of lamprey plasma. After 48 h of incubation at 37^{o}C, the cleared ring diameters around the wells due to the lytic action of lysozyme on *M. luteus* were measured in mm with a calibrating viewer. Lysozyme plasma protein and hen egg white lysozyme concentrations were both 1 μg/well with 10 μl being applied to an assay well.

The lamprey lysozyme concentration was compared with channel catfish (*Ictalurus punctatus*) lysozyme, done at the same time and using the same assay method. The channel catfish had a concentration of 222 ng lysozyme/mg total serum protein. A 1:200 dilution of lamprey plasma gave no clearing around wells for lysozyme activity.

The lamprey lysozyme was isolated from plasma using Bio-Gel P-10 polyacrylamide gel filtration column chromatography. The Bio-Gel P-10 gel has a fractionation range of 1,500-20,000 Daltons. 0.5 ml of the lamprey plasma was added to the column and eluted with 0.1 M NaH_2PO_4 buffer, pH 7.4. One milliliter fractions were collected and assayed for protein at 280 nm (Fig. 3.1). An absorbancy peak of 0.228 was identified for fraction number 27. Lysozyme well assay of column fraction #27, done in triplicate, showed activity against *M. luteus*, *B. megaterium*, and *M. phlei*. All three are Gram-positive species (Table 3.1). No bactericidal activity by fraction #27 was seen against the Gram-negative bacteria *A. salmonicida* and *E. coli* or against the two fungal species (Table 3.1).

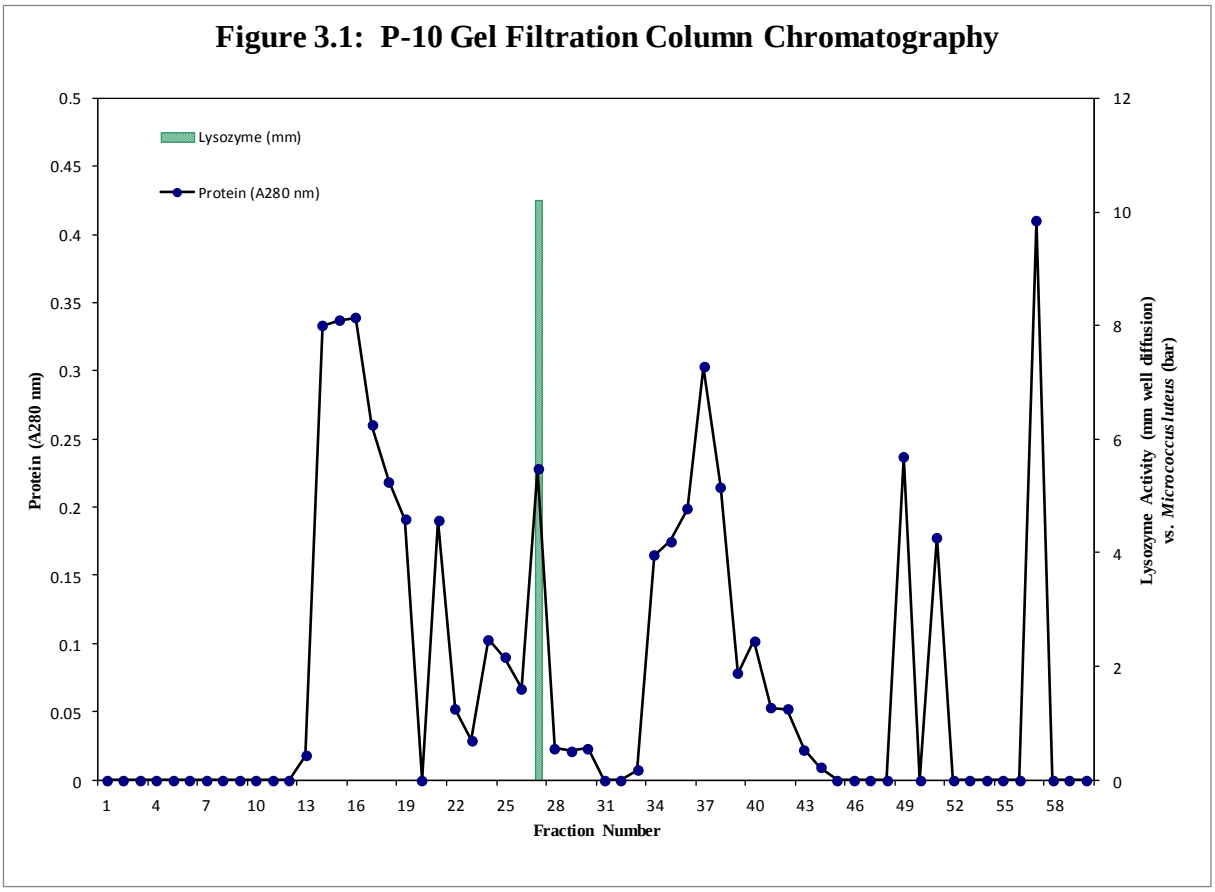

Figure 3. 1: Graph of protein absorbancies at 280 nm of eluted fractions from P-10 gel filtration column chromatography. Lysozyme activity was seen in fraction #27 (bar) against *Micrococcus luteus.*

Table 3. 1: Lysozyme activity using fraction #27 was determined by well diffusion assay against Gram-positive bacteria, Gram-negative bacteria and fungi. Antifungal activity using fraction #49 was determined by well diffusion assay against two fungal species.

Lysozyme Activity Assay

	Zone of Inhibition (mm)	
Bacteria and Fungi	Sea Lamprey Lysozyme*	Egg White Lysozyme**
Micrococcus luteus	12.9	15.6
Bacillus megaterium	9.0	9.2
Mycobacterium phlei	7.6	3.0
Aeromonas salmonicida	3.0	3.0
Escherichia coli	3.0	3.0
Penicillium notatum	3.0	3.0
Aspergillus flavus	3.0	3.0
Saline control	3.0	3.0

Note. Lysozyme activity was determined by well diffusion assay against Gram-positive bacteria, Gram-negative bacteria and fungi. The diameter of each well was 3 mm. 10 µl were applied to each well. Done in triplicate.

*Lamprey lysozyme (Fraction #27) from P-10 column chromatography

**Zone of clearing at concentration of 100 µg/ml of hen egg white lysozyme

Antifungal Plasma Activity Assay

	Zone of Inhibition (mm)		
Fungi	Sea Lamprey Plasma	Fraction # 49*	Control**
Penicillium notatum	11.0	12.5	3.0
Aspergillus flavus	11.2	9.5	3.0

Note. Antifungal activity was determined by well diffusion assay against two fungal species. The diameter of each well was 3 mm. 20 µl of sample were applied to each well. Done in triplicate.

*Fraction #49 from P-10 gel filtration column chromatography of plasma.

**0.1 M NaH_2PO_4, pH 7.4

The molecular weight of the purified lamprey lysozyme in fraction #27 from P-10 column chromatography was determined using an SDS-PAGE pre-cast 4-20% linear gradient Tris-HCL gel. 25 µl samples of fraction #27 and 100 µg/ml of hen egg white lysozyme were each mixed with 5 µl of sample loading buffer and then added to gel lanes along with the protein standards (Fig. 3.2). After electrophoresis for 90 min, the gel was placed in Coomassie brilliant blue to stain the protein bands. Fraction #27 and the hen egg white lysozyme both migrated just below the 15 kDa standard (Fig. 3.2). The hen egg white lysozyme has a molecular weight of 14.3 kDa. The molecular weight of the lamprey lysozyme, isolated in column fraction #27, had an approximate molecular weight of 14.3 kDa (Fig. 3.2).

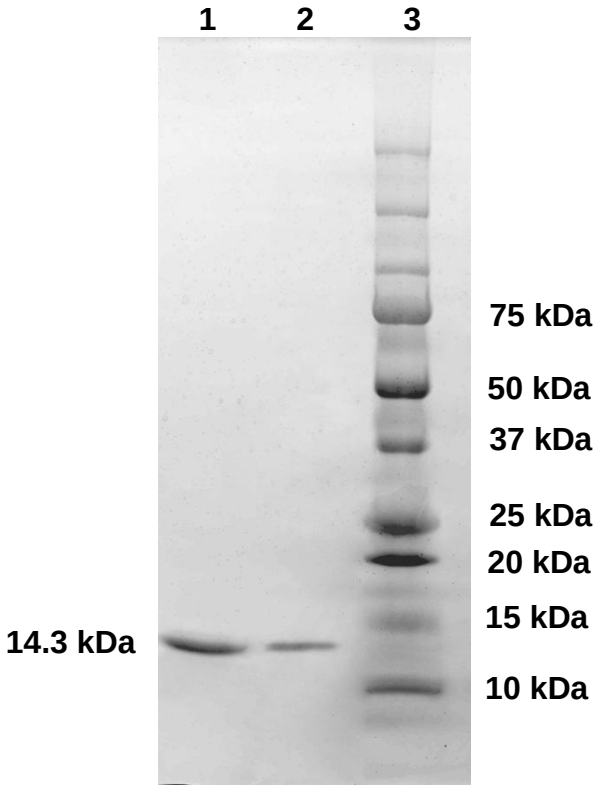

Figure 3.2. SDS-PAGE of Fraction #27 from P-10 (Bio-Rad) Column Chromatography. Lane 1: 100 µg/ml hen egg white lysozyme standard; Lane 2: Lamprey lysozyme (Fraction #27); Lane 3: Precision plus molecular weight standards (Bio-Rad). 30 µl samples were applied to Lanes 1 and 2.

The concentration of the isolated sea lamprey lysozyme was 195 µg/ml. This is similar to the 200 µg/ml concentration of the plasma lysozyme calculated using a standard curve of hen egg white lysozyme. All of the plasma lysozyme protein was apparently isolated by P-10 gel filtration chromatography (Fig. 3.1).

Antifungal plasma activity against *P. notatum* and *A. flavus* was observed (Table 3.1). The antifungal peptide was also isolated from the sea lamprey plasma using P-10 gel filtration chromatography. Antifungal activity was essentially confined to elution

fraction #49 from the P-10 gel filtration column (Fig. 3.3). Antifungal protein concentration (fraction #49 from gel filtration chromatography) was 306 µg/ml by BCA assay.

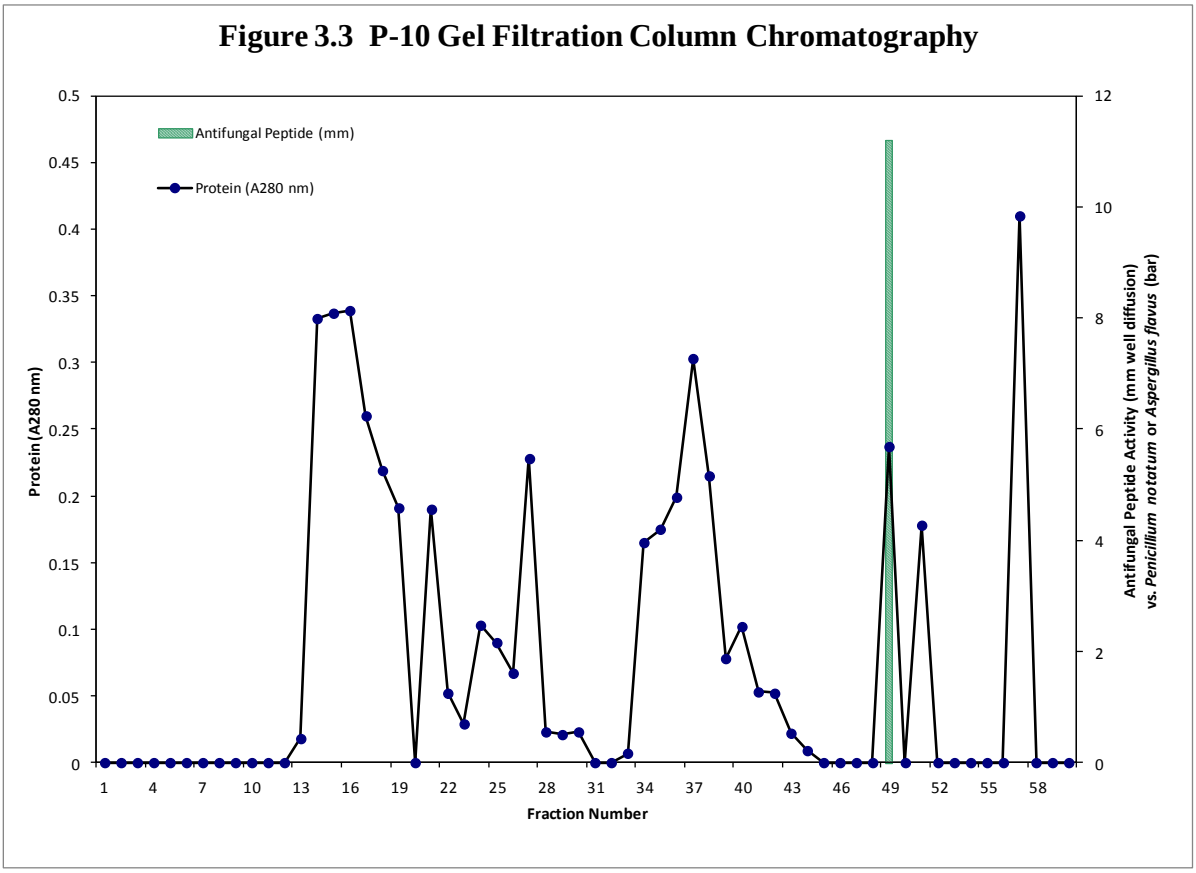

Figure 3.3: Graph of protein absorbencies at 280 nm of eluted fractions from P-10 gel filtration column chromatography. Antifungal peptide activity was seen in fraction #49 (bar) against *Penicillium notatum* and *Aspergillus flavus*.

To determine the molecular weight of the antifungal peptide, low molecular weight standards were eluted from the P-10 gel filtration column using the elution buffer, 0.1 M NaH_2PO_4, pH 7.4. Protein absorbancies were measured at 280 nm. Molecular weights and fraction numbers were plotted to prepare a standard curve from which the molecular weight of the antifungal peptide (fraction #49) was determined (approximately 3,000 Daltons).

Lukyanenko (1965) first reported the presence of lysozyme in fish serum. We previously reported the presence of lysozyme in channel catfish serum and skin mucus at a concentration of 34 ng lysozyme/mg protein and 46 ng lysozyme/mg protein, respectively (Ourth, 1980; Ourth and Wilson, 1981). Lysozyme is most effective in hydrolysis of the peptidoglycan cell wall of Gram-positive bacteria. If the outer membrane of Gram-negative bacteria is damaged by innate immune factors or the complement system, then lysozyme can also hydrolyze the peptidoglycan cell wall layer of Gram-negative bacteria (Ryan and Ray, 2004). Fish lysozyme may have a greater activity spectrum when compared with mammalian lysozyme, since it can be active against both Gram-positive bacteria and Gram-negative bacteria (most fish pathogens are here) (Watts et al., 2001). This may be due to the presence of different isoforms of fish lysozyme as found in rainbow trout (Grinde et al., 1988). The sea lamprey lysozyme isolated here (Fig. 3.2) was only effective against Gram-positive bacteria (Table 3.1) indicating a difference with the lysozymes of many teleost fish (Jolles and Jolles, 1984; Grinde et al., 1988).

The lamprey plasma and lysozyme were most effective against *M. luteus*. Lysozyme was also effective against *B. megaterium* and *M. phlei* (Table 3.1).

Mycobacterial species are important pathogens of many teleost fish including striped bass of the Chesapeake Bay area USA. Mycobacteriosis is of concern to commercial fishermen in the Chesapeake Bay, as one species of *Mycobacterium* is a zoonotic agent that can potentially infect humans (Ottinger et al., 2003).

Antifungal plasma activity against *P. notatum* and *A. flavus* was seen (Table 3.1). Fraction #49 (Fig. 3.3) from P-10 gel filtration chromatography indicated antifungal activity. The antifungal clearing around the well, using 6 µg/well of sea lamprey antifungal peptide, was nearly equivalent to antifungal susceptibility testing when using 25 µg/well of known antifungal azole compounds (Magaldi et al., 2004).

Antifungal peptides, like the defensins, have been identified in insects and mammals (Andra et al., 2001; Zasloff, 2000; Sahl and Bierbaum, 2008). α-Defensins are found in granules of neutrophils and have antifungal and antibacterial activity. α-Defensins are pore-forming, low molecular weight peptides active against fungal cell membranes including *A. flavus* and *A. fumigatus* (DeLucca and Walsh, 1999 and 2000). Antifungal activity by the peptide Cecropin A has also been found against *Aspergillus* species (DeLucca et al., 1997). Fungal species are widely found in the environment including soil and decaying organic matter.

The lamprey plasma was not effective against *E. coli* or *A. salmonicida* which indicates that lysozyme does not have lytic activity against Gram-negative bacteria. The eluted fractions from P-10 column chromatography also showed no bactericidal activity against *E. coli* or *A. salmonicida*. This indicates the absence of antibacterial peptides in both plasma and P-10 fractions (Chung and Ourth, 2000; Ourth and Chung, 2004). Yun and Li (2007) also did not find antibacterial peptides in sea lamprey leukocytes**.** No

innate immunity to *A. salmonicida* was found, indicating that this bacterial species could serve as a potential pathogen in the lamprey natural environment (Ourth et al., 2008).

The molecular weight of the sea lamprey lysozyme isolated here (14.3 kDa) was equivalent to hen egg white lysozyme (Fig. 3.2). This compares with 14.4 kDa for rainbow trout (*Oncorhynchus mykiss*) lysozyme (Grinde et al., 1988), 14.5 kDa for coho salmon (*Oncorhynchus kisutch*) lysozyme (Yousif et al., 1991), and 14.5 kDa for Atlantic salmon (*Salmo salar*) lysozyme (Fagan et al., 2003).

Lysozyme was detected in the sea lamprey plasma pool at a concentration of 5 μg lysozyme/mg total protein. When compared with channel catfish lysozyme determined at the same time (222 ng lysozyme/mg total protein), the sea lamprey was 23-times greater in lysozyme concentration than the channel catfish lysozyme. The large difference in lysozyme concentration between sea lamprey and channel catfish may be because the channel catfish, an advanced teleost fish, has both innate immunity and adaptive immunity. In contrast, the sea lamprey which is the most primitive fish, has only innate immunity and may therefore compensate by producing a much greater concentration of plasma lysozyme when compared with the channel catfish. Lysozyme has been found in many fish species and now here in sea lamprey plasma.

Squalamine, a non-peptide antimicrobial factor of innate immunity, has been found in white blood cells of the sea lamprey (Yun and Li, 2007). A squalamine-like compound (655 Daltons) was identified in channel catfish leukocytes and may play a role in innate immunity (Ourth and Chung, 2004). Mannose-binding lectin, an important component of innate immunity (Kindt et al., 2007) has been previously identified and

characterized in this sea lamprey species (Ourth et al., 2008) and also in the channel catfish (Ourth et al., 2007).

Lysozyme and antifungal peptides are important components of innate immunity in animals (DeLucca and Walsh, 1999 and 2000; Kindt et al., 2007). The data indicate that the high concentration of both plasma lysozyme and antifungal peptide as found here could be very important in sea lamprey resistance to bacterial and fungal infections. Fungal infections must be important in lamprey health and innate immunity, since this sea lamprey species produces its own antifungal peptide.

Acknowledgement

We thank Dr. Michael J. Siefkes, U.S. Fish and Wildlife Service, Marquette Biological Station, Marquette, MI for providing the sea lamprey plasma.

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Chapter 4: Effects of Suppression by Prednisolone Acetate on Innate and Serum Proteins in Ictalurid Catfish

1. Introduction

The channel catfish is extensively used in aquaculture in the Southeastern USA. Catfish can be subjected to stressful pond conditions which contribute to fish morbidity and mortality (Small and Bilodeau, 2005). Stress can be physical, such as overcrowding, poor water quality and handling, or of a chemical nature (Woo and Bruno, 2009). The onset of stress leads to an increase in circulating plasma cortisol and glucose or the fight-or-flight response. Cortisol is a glucocorticoid and the primary component of the stress response in fish. In teleost fish, cortisol is also important in metabolism, growth and osmoregulation (Mommsen et al., 1999).

Studies have been done correlating the effects of acute stress on the immune response of catfish (Ellsaesser and Clem, 1987; Bilodeau et al., 2005; Small and Bilodeau, 2005). These studies show that short-term or acute stress may even be beneficial to the immune health of farmed fish, stimulating both innate and adaptive immune responses for better disease resistance (Maule et al., 1989; Demers and Bayne, 1997; Bilodeau et al., 2003; Bilodeau et al., 2005). With acute or short-term stress, the increase in cortisol and glucose is only temporary. Long-term elevation in plasma cortisol associated with chronic stress can lead to immunosuppression and higher rates of infection in fish (Ellsaesser and Clem, 1987; Bilodeau et al., 2005).

Published research has shown that continuous exogenous cortisol administration through cortisol-treated feed caused elevated plasma cortisol levels in channel catfish that returned to normal within 24 hrs (Small and Bilodeau, 2005). It was determined that

exogenous cortisol had very little effect on disease susceptibility of channel catfish. On the other hand, a stress event followed by exposure of channel catfish to a pathogen, bacterial *Edwardsiella ictaluri* or protozoan parasite *Ichthyophthirius multifiliis*, did increase disease susceptibility of the fish (Davis et al., 2002; Small and Bilodeau, 2005). This would indicate that stress, but not exogenous cortisol, has immunosuppressive effects.

In this study, the stress response was chemically-induced by injecting catfish with prednisolone acetate (PA) which mimics cortisol (McCarthy, 1977). PA has been used in other studies to induce a stress response and suppress immunity to reveal latent infections (Hiney et al., 1994; Cipriano et al., 1997). PA is like cortisol in that it is a potent glucocorticoid; however, PA is also a modest mineralocorticoid as well. Both Hiney (1994) and Cipriano (1997) injected PA into Atlantic salmon and brook trout at a rate of 20mg/kg to reveal latent bacterial infections. In both experiments, fish that survived for 14 days were euthanized. No plasma or serum was tested to determine the effects of this chemical stressor other than to determine which fish were bacterially infected.

The effects of chemical stress by PA on serum protein levels and two proteins of the innate immune response, lysozyme and mannose-binding lectin (MBL), have not before been reported in this Ictalurid hybrid aquaculture species and is of interest here. The fish immune system is considered a crossroads between the innate and adaptive immune responses (Tort et al., 2003). This means that fish may be even more dependent on innate immunity than other vertebrates.

2. Methods and materials

2.1. Experimental design

The first objective of this research was to acclimate and then pre-bleed 60 Ictalurid catfish to establish their base-line levels for total protein, albumin, globulin, MBL, lysozyme and glucose. Because the stress response varies from species to species and even from fish to fish, it is important to bleed each fish and track each fish separately throughout the experiment. All 60 fish were anesthetized and bled each week. The serum of each of the 60 catfish was tested each week for total protein, albumin, MBL and lysozyme. A weekly comparison was made then for each fish.

2.2. Maintenance of catfish

Adult hybrid catfish (blue catfish D+B x channel catfish NWAC 103) were maintained in tanks at 27°C in a re-circulating water-reuse culture system at the Ecological Research Center, Department of Biological Sciences, University of Memphis (Dr. Bill Simco, Director). Sixty adult catfish (3 years-old) were acclimated in 10 tanks (6 fish/tank) for four weeks before PA injection. Tanks (400 gallon) were monitored for optimal water quality conditions and all catfish were fed the same diet (floating catfish pellet food, Rangen, Inc., Buhl, ID, EXTR 450). Fish were marked for recognition.

2.3. Serum collection and injection

After acclimation, the 60 catfish were pre-bled. Fish were anesthetized with 100 ppm tricaine methanesulfonate (MS 222, Argent Chemical Laboratories, Redmond, WA) before each bleeding. Fish were unconscious within 90 seconds and bleeding was done within one minute. Blood was collected from the caudal blood sinus of each catfish using

heparinized needles. The blood was allowed to clot and serum was separated by centrifugation. The sera obtained were stored at -80°C (Ourth and Wilson, 1982; Ourth et al., 2007).

One week after the pre-bled sera were collected, thirty catfish were intraperitoneally (IP) injected with prednisolone acetate (PA, Sigma Chemical, St Louis, MO) at a dosage of 20 mg PA/kg of fish weight to induce a stress response (McCarthy, 1977; Hiney et al., 1994; Cipriano et al., 1997). Thirty control catfish were sham-injected IP with saline. Blood samples were collected from each of the sixty catfish for up to five weeks post-injection.

2.4. Serum protein determinations

Total protein concentrations of serum samples from each fish were determined using the BCA protein assay (Pierce Chemical, Rockford, IL). Bovine serum albumin was used as the protein standard. Albumin concentrations were determined using an assay kit from Pointe Scientific (Canton, MI). Total globulin concentrations were determined by subtracting the albumin concentration from the total protein concentration. The albumin/globulin (A/G) ratios were then calculated (Ourth et al., 1991).

2.5. Lysozyme assay

Lysozyme activity for each serum sample was determined using a turbidimetric assay. Hen egg white lysozyme standards (50 µg/ml, 25µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.56 µg/ml and 0.78 µg/ml) were prepared using a 0.1 M phosphate citrate/citrate buffer, pH 5.8. The buffer was also used to prepare a suspension of *Micrococcus luteus*. Twenty-five microliters of undiluted fish serum were pipetted into wells of a 96-well plate. 175 µl of the *M. luteus* suspension were then added to each

well. Changes in turbidity were measured immediately and after five minutes on a microplate reader at 450 nm with rapid mixing (Demers and Bayne, 1997).

2.6. Enzyme-linked immunosorbent assay (ELISA) Dot-Blot for catfish mannose-binding lectin

A dot-immunoblot ELISA assay was performed using a dot-blot microfiltration apparatus to determine levels of mannose-binding lectin (MBL) in each of the catfish serum samples. The dot-blot apparatus (Bio-Rad, Richmond, CA) was used according to the manufacturer's immunoassay protocol and authors' previous work using this technique (Ourth et al., 2007a, 2007b; Ourth et al., 2008). The primary antibody was guinea pig antirabbit-MBL IgG. Rabbit anti-guinea pig IgG-horseradish peroxidase conjugate (Sigma Chemical, St Louis, MO) was added as the secondary antibody. A 3,3'-diaminobenzidine (DAB) solution was used to develop the brown colored product for 5 min and the reaction stopped with water (Ourth et al., 2007a, 2007b; Ourth et al., 2008).

2.7. Glucose concentration

Catfish sera were analyzed for glucose concentrations using a Stat Profile Critical Care Xpress blood gas analyzer (Novo Biomedical, Waltham, MA) at the USDA Catfish Genetics Research Unit, Stoneville, MS (Beecham et al., 2010).

2.8. Statistical analysis

Statistical significance was determined by paired difference or "repeated measures" two sample t-test using SPSS Statistics v20 (SPSS, IBM software, IBM.com). Statistical comparisons of weekly concentrations of total protein, albumin, globulin, glucose, mannose-binding lectin and lysozyme for each fish were determined (Ourth et

al., 1991). A comparison of mortality between sham-injected catfish and PA-injected catfish will be done using Fisher's exact test.

3. Results

3.1. Sham-injected control catfish

Thirty catfish were sham-injected IP with saline. These 30 sham-injected catfish were held in the same re-circulating water-reuse culture system as the 30 PA-injected catfish. The 30 sham-injected catfish were also anesthetized, bled each week and subjected to the same handling stress as the PA-injected catfish. Sham-injected control catfish showed no significant increase or decrease in total protein concentration, lysozyme or MBL levels when compared with their pre-bled sera levels.

3.2. Survival of catfish

A comparison was made between the 30 PA-injected catfish and the 30 sham-injected control catfish based on mortality levels. Of the 30 PA-injected catfish, only 10 survived for the full five weeks after PA-injection. All 30 of the control sham-injected catfish survived for the full five weeks after saline-injection. A Fisher's exact test using a 2X2 contingency table revealed a two-tailed *P* value of less than 0.001. All 60 of these catfish were subjected to the same water quality, feed type, and handling stress (weekly bleeding and IP injection). The only difference was the IP-injection with either PA or saline.

3.3. Groupings of PA-injected catfish

Over five weeks, the 30 PA-injected catfish were bled and their sera individually assayed each week. Table 4.1 contains the protein and glucose determinations of all 30 PA-injected catfish for the six-week experimental period. Table 4.2 contains the

concentrations of the two innate immune proteins, lysozyme and MBL. Table 4.2 also includes the 30 PA-injected catfish over the six-week experimental period.

For presentation of the data, the serum samples of the 10 catfish that survived all six weeks of the experimental period, which includes the five weeks post-injection with PA, are in Group 1. The serum samples of the 10 catfish that survived for only four weeks, which includes three weeks post-injection with PA, are in Group 2. The serum samples of the 10 catfish that survived for two weeks, which includes one week post-injection with PA, are in Group 3.

Table 4.1: Total protein, albumin, globulin, A/G ratio and glucose determinations over a six-week period of the three groups of catfish injected once with prednisolone acetate (PA). Week 1 reflects pre-bled serum concentrations before PA was injected.

Table 4.1: Protein and Glucose Determinations

	Week 1 (Pre-Bled)			Week 2			Week 3		Week 4		Week 5	Week 6
Group[1]	G1	G2	G3	G1	G2	G3	G1	G2	G1	G2	G1	G1
Total Protein[2]	38.8	28.2	26.6	26.4	18.9	18.3	22.6	16	21.5	15.1	20.7	18.3
Albumin[2]	9.6	12.2	11	8.3	12.5	10.1	6.1	10.8	6	8.4	11.2	11.6
Globulin[2]	29.4	16	15.6	18.2	6.2	8.2	16.5	6.2	15.6	6.7	9.5	6.7
A/G Ratio	0.33	0.76	0.71	0.46	2.02	1.23	0.37	1.74	0.39	1.25	1.18	1.73
Glucose[3]	80	71	75	141	124	83	146	153	157	109	132	101

[1]Each Group (G) is the mean of 10 catfish.

[2]Protein concentrations in mg/ml

[3]Glucose concentrations in mmol/l

Injection of prednisolone acetate (PA) occurred one week after pre-bleeding. Week 2 sera were taken one week after PA injection.

Group 1 (G1) catfish survived 5 weeks after injection with PA.

Group 2 (G2) catfish survived 3 weeks after injection with PA.

Group 3 (G3) catfish survived 1 week after injection with PA.

Table 4.2: Lysozyme and mannose-binding lectin determinations over a six-week period of the three groups of catfish injected once with prednisolone acetate (PA). Week 1 reflects pre-bled serum concentrations before PA was injected.

Table 4.2: Innate Immune Proteins

	Week 1 (Pre-Bled)			Week 2			Week 3		Week 4		Week 5	Week 6
Group[1]	G1	G2	G3	G1	G2	G3	G1	G2	G1	G2	G1	G1
Lysozyme[2]	5.6	4.3	3.9	6.1	4.8	4.4	4.25	3.1	4.1	2.3	3.9	2.9
Mannose Binding Lectin[3]	53.1	39.8	24.65	75.4	62	30.2	53.9	35.5	22.2	12.1	20.9	18.4

[1]Each Group (G) is the mean of 10 catfish.

[2]µg/ml

[3]Background-corrected inverse density units by Image J Analysis

Phosphate citrate/citrate buffer control used for lysozyme turbidimetric assay

Tris-buffered saline control used in dot-blot ELISA mannose-binding lectin assay

Injection of prednisolone acetate (PA) occurred one week after pre-bleeding. Week 2 sera were taken one week after PA injection.

Group 1 (G1) catfish survived 5 weeks after injection with PA.

Group 2 (G2) catfish survived 3 weeks after injection with PA.

Group 3 (G3) catfish survived 1 week after injection with PA.

3.4. Total protein concentration

Total protein concentrations for the three groups of catfish decreased significantly ($P<0.001$) over the six-week period (Table 4.1). Both Groups 1 and 2 decreased by approximately 50% when compared with their pre-bled serum total protein concentrations (Fig. 4.1).

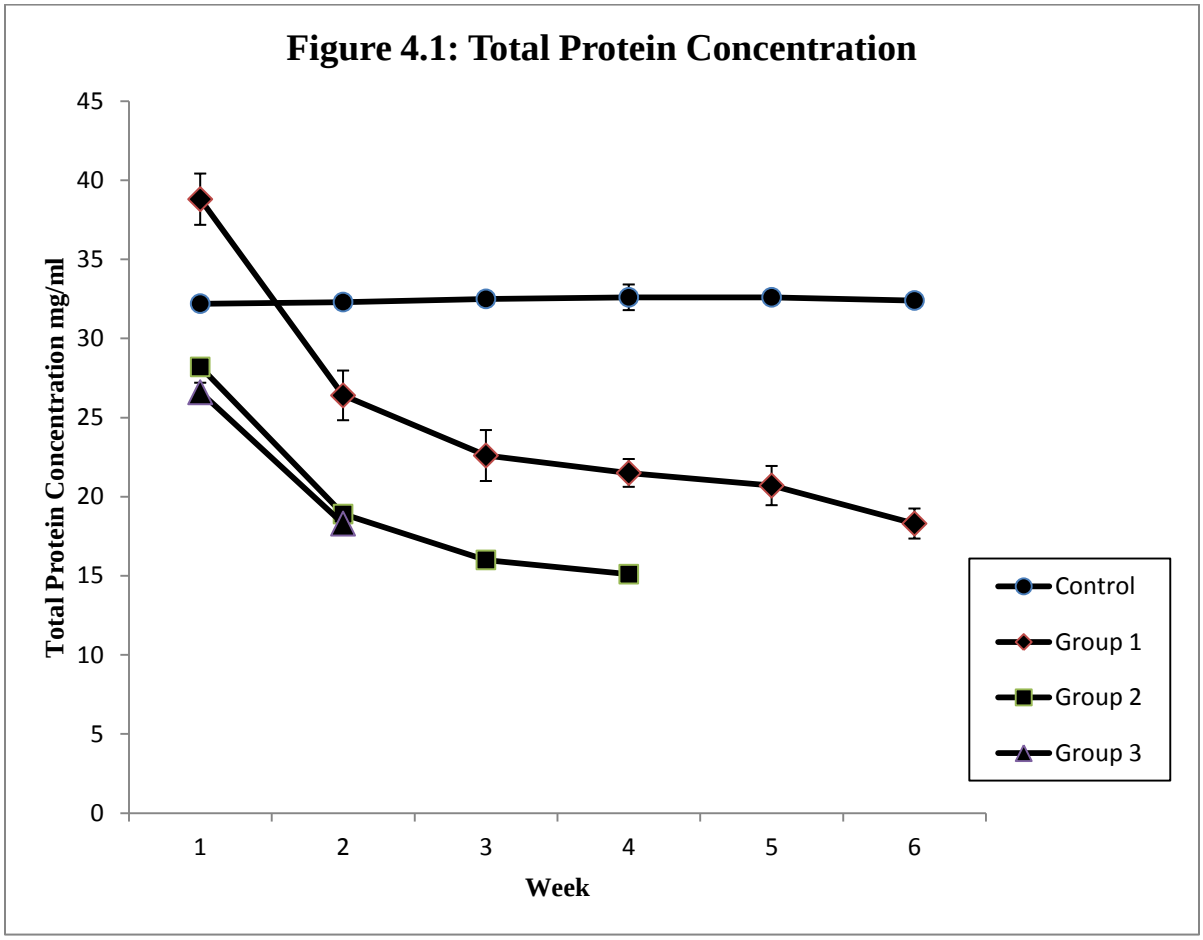

Figure 4.1: Graph of total protein concentrations over a six-week period of the three groups of catfish injected once with prednisolone acetate (PA). Week 1 reflects the pre-bled serum total protein concentrations before PA was injected. Error bars reflect +/- SEM.

The serum protein levels of the control sham-injected catfish did not significantly differ when compared with their pre-bled sera indicating no long-term suppressive effects in protein synthesis (Fig. 4.1). The effects of acute handling stress on ictalurid catfish have been previously reported in that total serum protein levels usually returned to within normal range within 30 minutes after handling stress (Ellsaesser and Clem, 1987).

The pre-bled sera for the control catfish, to be sham-injected, assayed at 32.2 mg/ml total protein (range of 32 to 32.4 mg/ml protein for the 30 control fish). One week after IP injection with saline, the total protein concentration of the sham-injected control catfish was 32.3 mg/ml (range of 32.1 to 32.6mg/ml protein). Two weeks after IP injection with saline, the total protein concentration of the sham-injected control catfish was 32.5 mg/ml (range of 32.0 to 32.7 mg/ml protein). Three weeks after IP injection with saline, the total protein concentration of the sham-injected control catfish was 32.6 mg/ml (range of 31.2 to 34 mg/ml protein). Four weeks after IP injection with saline, the total protein concentration of the sham-injected control catfish was 32.6 mg/ml (range of 32.5 to 32.6 mg/ml protein). Five weeks after IP injection with saline, the total protein concentration of the sham-injected control catfish was 32.4 mg/ml (range of 32 to 32.6 mg/ml protein) (Fig 4.1).

3.5. Albumin concentration

Albumin concentrations decreased slightly for the three groups of catfish in the first three weeks after injection with PA (Table 4.1). In Group 1, the albumin concentration increased significantly ($P<0.001$) at weeks five and six (Fig.4.2).

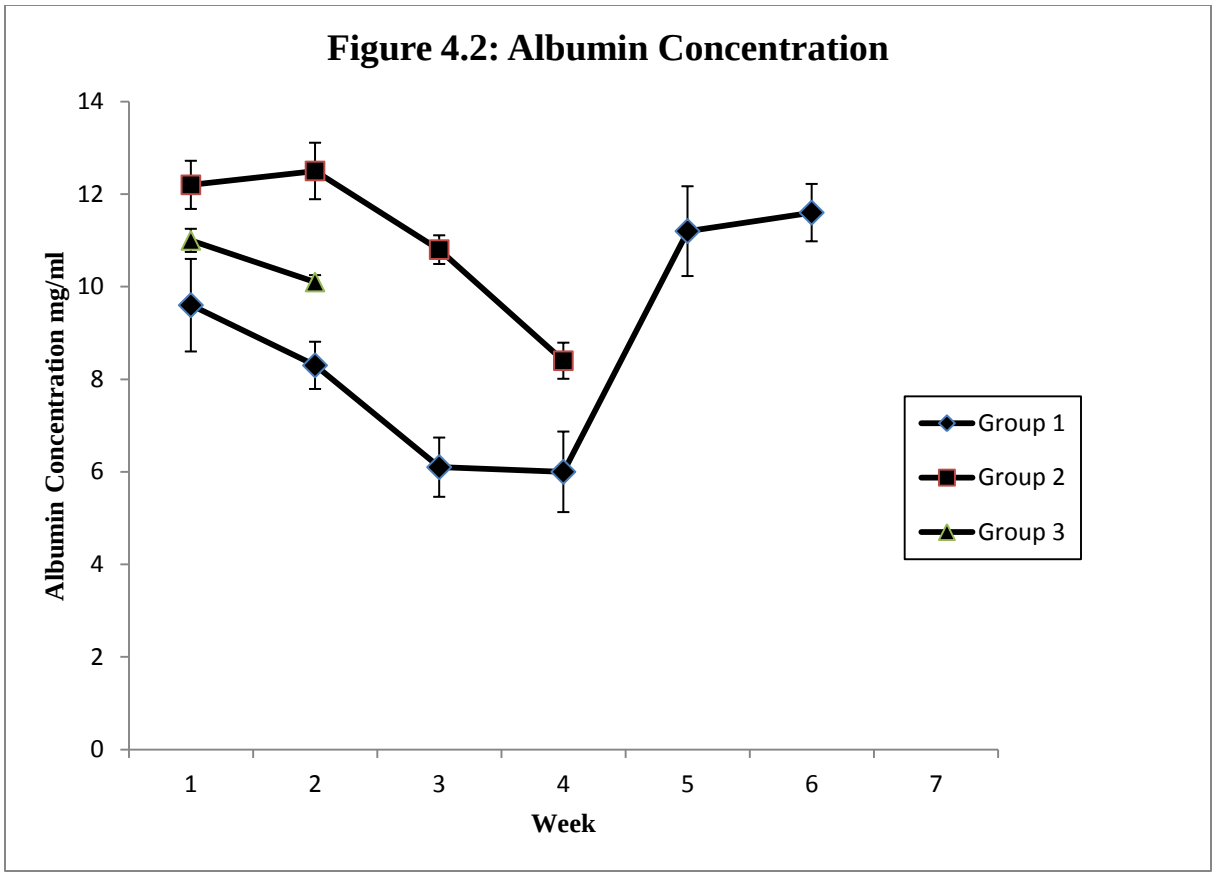

Figure 4.2: Graph of albumin concentrations over a six-week period of the three groups of catfish injected once with prednisolone acetate (PA). Week 1 reflects the pre-bled serum albumin concentrations before PA was injected. Error bars reflect +/- SEM.

3.6. Globulin concentration

Globulin concentrations decreased significantly ($P<0.001$) at one week following PA injection for all three groups (Table 4.1). By week six, the globulin concentration for Group 1 had decreased by four-times compared with its pre-bled serum globulin concentration (Fig. 4.3).

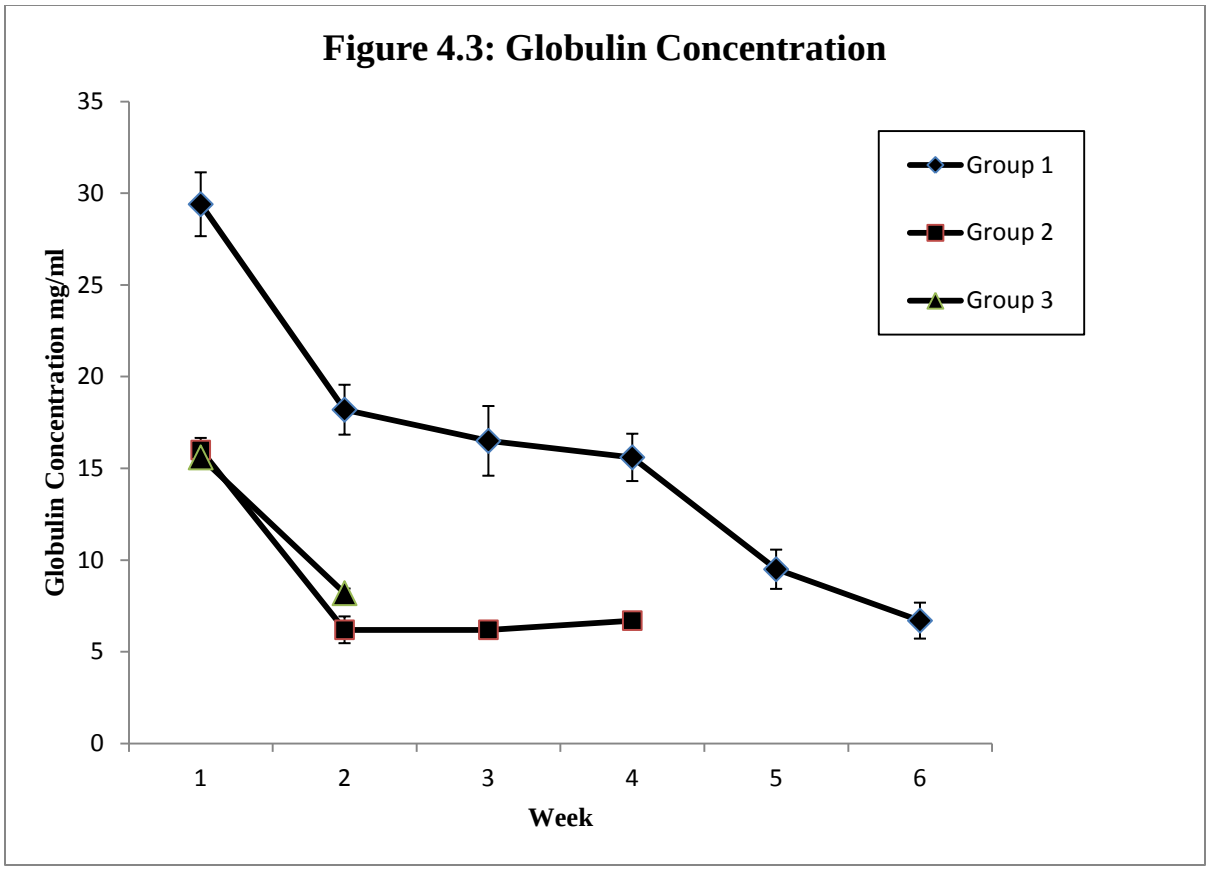

Figure 4.3: Graph of calculated globulin concentrations over a six-week period of the three groups of catfish injected once with prednisolone acetate (PA). Week 1 reflects the pre-bled serum globulin concentrations before PA was injected. Error bars reflect +/- SEM.

3.7. Albumin/Globulin (A/G) ratio

The A/G ratio for Group 1 remained consistent for three weeks post-injection with PA and then significantly increased ($P<0.001$) five times when compared with the pre-bled serum A/G ratio (Table 4.1). The A/G ratios for Groups 2 and 3 increased significantly the week following injection with PA (Table 4.1).

3.8. Glucose concentration

The glucose concentrations increased for the three groups of catfish following injection with PA (Table 4.1). The glucose concentration continued to increase for Groups 1 and 2 for weeks two and three. The glucose concentration for Group 2 decreased significantly (P<0.001) in week four (Fig. 4.4). In Group 1 catfish, the glucose concentration decreased in week five but did not decrease significantly (P<0.001) until week six, which is five weeks post-injection with PA (Fig. 4.4).

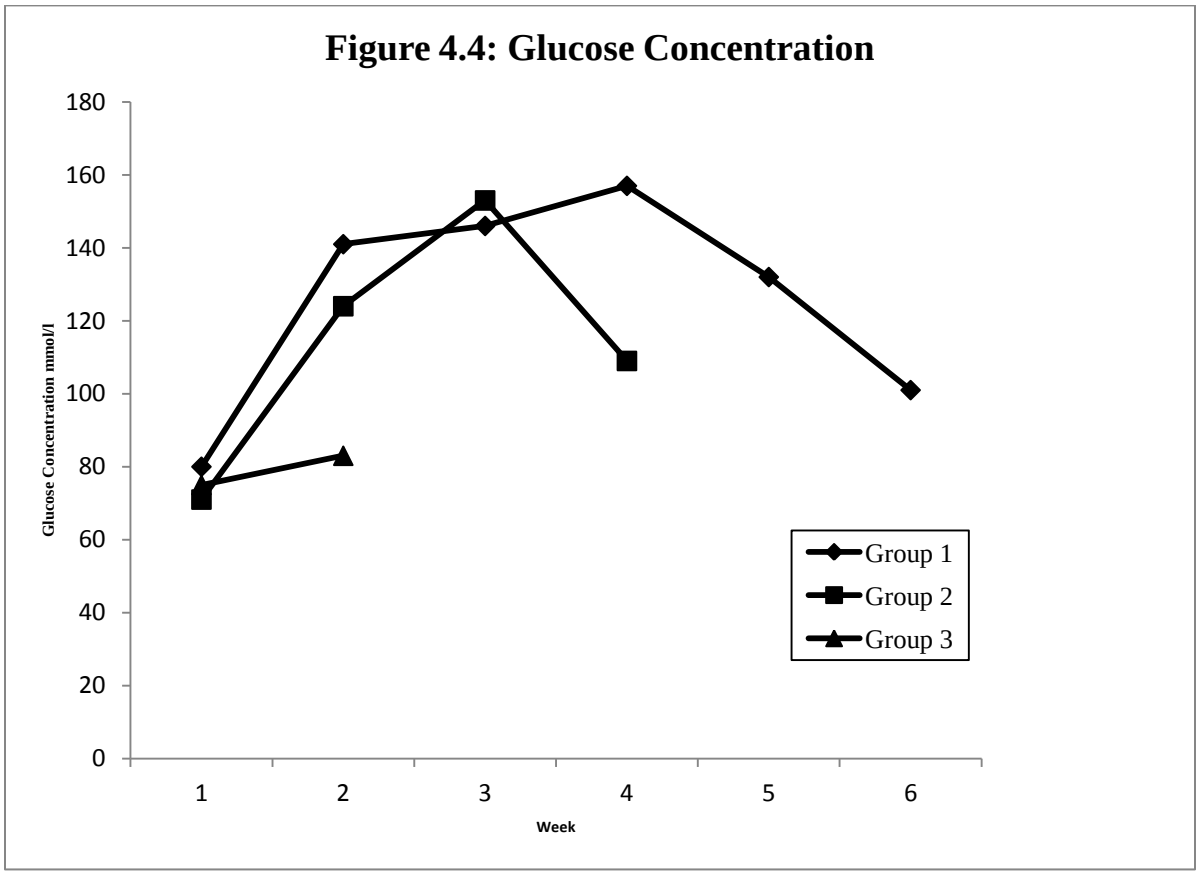

Figure 4.4: Graph of glucose concentrations over a six-week period of the three groups of catfish injected once with prednisolone acetate (PA). Week 1 reflects the pre-bled serum glucose concentrations before PA was injected.

3.9. Lysozyme concentration

The lysozyme concentrations increased for all three groups the week following the PA injection (Table 4.2). The lysozyme concentrations for both Groups 1 and 2 decreased significantly ($P<0.001$) by 50% at week four when compared with the pre-bled serum lysozyme concentrations (Fig. 4.5).

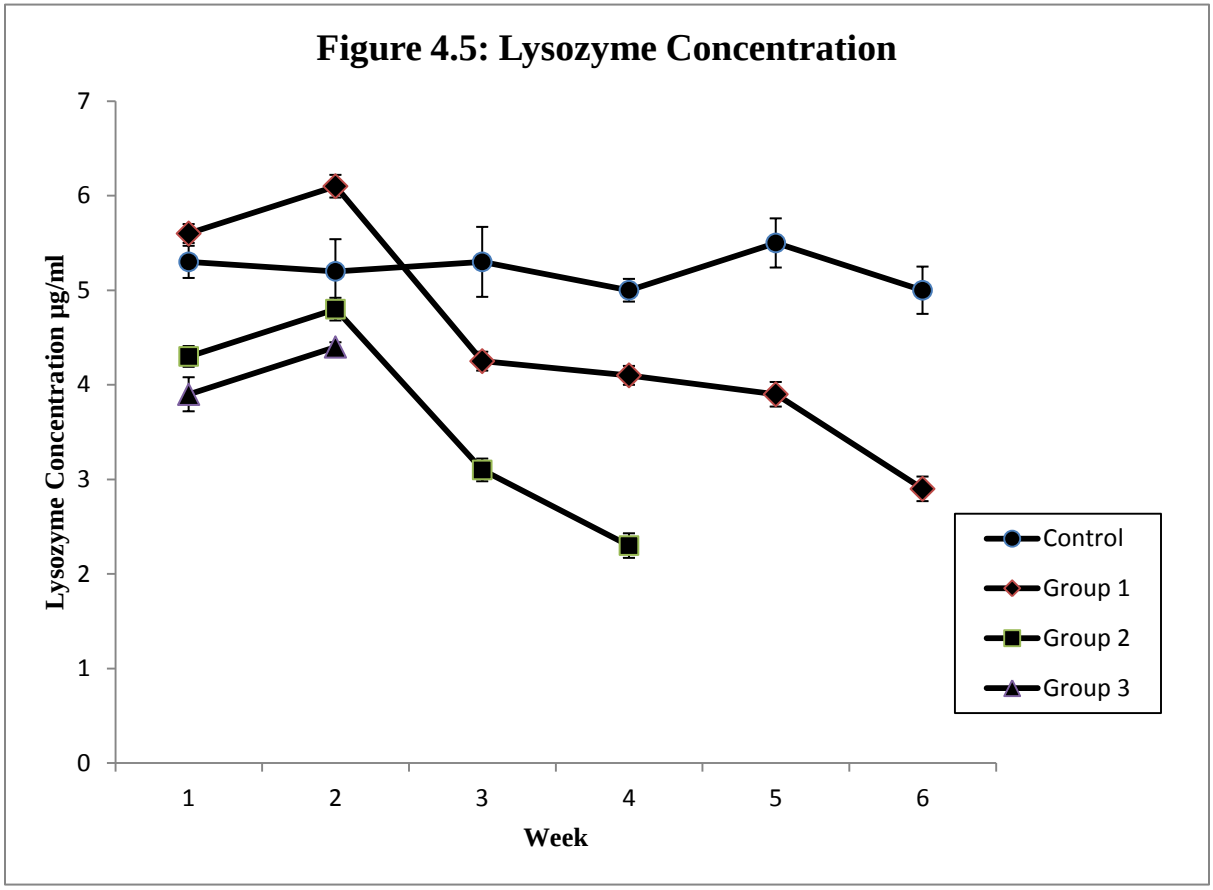

Figure 4.5: Graph of lysozyme concentrations over a six-week period of the three groups of catfish injected once with prednisolone acetate (PA). Week 1 reflects the pre-bled serum lysozyme concentrations before PA was injected. Error bars reflect +/- SEM.

The sham-injected control catfish showed no significant changes in lysozyme activity when compared with pre-bled sera activity levels (Fig. 4.5). Demers and Bayne (1999) previously reported that serial bleeding and handling stress without an intentional acute stressor did not result in an increase in plasma lysozyme.

The pre-bled sera for the control catfish, to be sham-injected, assayed at 5.2 µg/ml of lysozyme (range of 5 to 5.5 µg/ml lysozyme for 30 control catfish). One week after IP injection with saline, the lysozyme concentration of the sham-injected control catfish was 5.2 µg/ml (range of 4.8 to 5.9 µg/ml lysozyme). Two weeks after IP injection with saline, the lysozyme concentration of the sham-injected control catfish was 5.3 µg/ml (range of 4.8 to 6 µg/ml lysozyme). Three weeks after IP injection with saline, the lysozyme concentration of the sham-injected control catfish was 5 µg/ml (range of 4.8 to 5.2 µg/ml lysozyme). Four weeks after IP injection with saline, the lysozyme concentration of the sham-injected control catfish was 5.5 µg/ml (range of 5 to 5.9 µg/ml lysozyme). Five weeks after IP injection with saline, the lysozyme concentration of the sham-injected control catfish was 5 µg/ml (range of 4.7 to 5.5 µg/ml lysozyme) (Fig 4.5).

3.10. Mannose-binding lectin (MBL) concentration

The MBL concentrations increased significantly ($P<0.001$) for all three groups of catfish the week following the PA injection (Table 4.2). The MBL concentrations for both Groups 1 and 2 decreased significantly ($P<0.001$) by 42% when compared with their pre-bled serum MBL concentrations (Fig. 4.6).

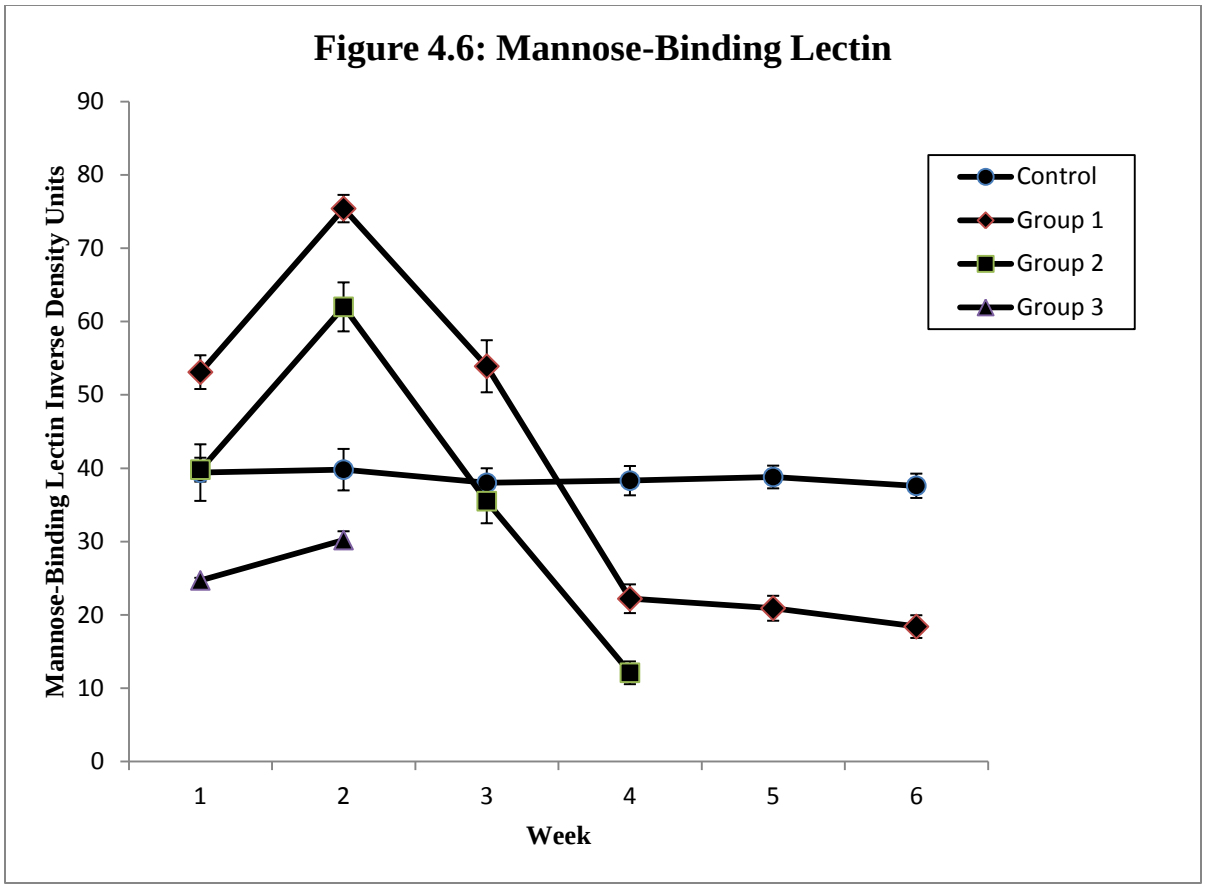

Figure 4.6: Graph of mannose-binding lectin (MBL) determinations over a six-week period of the three groups of catfish injected once with prednisolone acetate (PA). Week 1 reflects the pre-bled serum MBL determinations before PA was injected. Error bars reflect +/- SEM.

The sham-injected control catfish showed no significant change in MBL concentrations when compared with their pre-bled sera concentrations. The pre-bled sera for the control catfish, to be sham-injected, assayed at 39.4 inverse density units of MBL (range of 31.8 to 44.5 inverse density units for 30 control catfish). One week after IP injection with saline, the MBL level of the sham-injected control catfish was 39.8 inverse density units (range of 36.4 to 45.4 inverse density units). Two weeks after IP injection with saline, the MBL level of the sham-injected control catfish was 38 inverse density

units (range of 35.9 to 41.6 inverse density units). Three weeks after IP injection with saline, the MBL level of the sham-injected control catfish was 38.3 inverse density units (range of 35.2 to 40.3 inverse density units). Four weeks after IP injection with saline, the MBL level of the sham-injected control catfish was 38.8 inverse density units (range of 36.4 to 41.6 inverse density units). Five weeks after IP injection with saline, the MBL level of the sham-injected control catfish was 37.6 inverse density units (range of 35.5 to 40.8 inverse density units) (Fig. 4.6).

4. Discussion

Stress induced by PA injection significantly decreased protein synthesis in the three groups of catfish. The ten catfish in Group 1 survived for all six weeks of the study. These ten catfish also had the highest concentrations of total protein before PA injection (Table 4.1). Group 1 catfish also had the highest pre-bled globulin, lysozyme and MBL concentrations (Tables 4.1 and 4.2). Group 2 catfish had the highest pre-bled albumin concentration and survived for four weeks, which includes three weeks post-injection with PA (Table 4.1).

The globulin concentrations in Table 4.1 may indicate the concentrations of IgM globulin for the three groups of catfish. By end of the study, Group 1 globulin concentration, which contained the highest pre-bled level of globulin, had decreased four-times and this could possibly indicate a decrease in fish IgM globulin (Table 4.1). This indicates that a long-term response to chemical stress could also negatively affect the adaptive immune response of catfish. Sahoo and Mukherjee (2001) demonstrated an immunosuppressive effect of chronic stress on total protein and globulin synthesis but not albumin synthesis in Indian major carp (*Labeo rohita*) after IP injection with aflatoxin B_1.

Albumin/globulin ratios can be used as an indicator of overall health in fish (Ourth et al., 1991; Sahoo and Mukherjee, 2001). Ourth et al. (1991) found healthy A/G ratios for channel catfish. They found that a ratio of approximately 0.333 indicated a healthy, normal three-year old channel catfish. The A/G ratio of catfish in Group 1 before PA injection was also 0.33. The Group 1 catfish here survived PA injection and the stress response for all six weeks. This indicates that A/G ratios could potentially be used for selecting long-term survival of channel catfish used in aquaculture.

The initial increase one week after PA injection in the concentrations of the innate immune proteins lysozyme and MBL (Table 4.2) supports the concept that acute or short-term stress can be beneficial in catfish immunity (Maule et al., 1989; Demers and Bayne, 1997; Bilodeau et al., 2003, 2005). However, two weeks after injection with PA, the concentrations of both lysozyme and MBL decreased significantly ($P<0.001$) in Groups 1 and 2 indicating that the prolonged or chronic effects of stress could be detrimental to catfish innate immunity. The innate immune response is of prime importance in fish (Tort et al., 2003).

Bilodeau et al. (2005) found that lysozyme activity in channel catfish with susceptibility differences to *Edwardsiella ictaluri* remained elevated in both susceptible and resistant catfish families for 20 days following challenge with the bacterium. The only difference was the onset of lysozyme activity. The resistant family of channel catfish had elevated lysozyme activity beginning one day after challenge with *E. ictaluri*. The susceptible family did not have elevated lysozyme activity until day two. They did not find that their data supported a link between serum cortisol and lysozyme activity. However, Yin et al. (1995) found that chronic stress caused by over-crowding in fancy

carp (*Cyprinus carpio*) led to an overall decrease in lysozyme activity. Lysozyme activity has been shown to increase and decrease depending on many variables, including toxicants and levels of stress (Saurabh and Sahoo, 2008). Lysozyme activity here was initially elevated one week after PA injection but activity decreased dramatically by the end of the week 6 (Table 4.2, Fig. 4.5). Lysozyme is therefore an important indicator of the innate immune response in fish.

The occurrence of *E. ictaluri* infections in pond aquaculture of channel catfish can be stress-related and is well documented (Woo and Bruno, 1999; Bilodeau et al., 2003; Bilodeau et al., 2005; Small and Bilodeau, 2005). Stressors in ponds include stocking densities of catfish, oxygen levels, ammonia, nitrite and temperature. Reducing the stress response of catfish can help in preventing enteric septicemia of catfish and other bacterial and microbial diseases (Woo and Bruno, 1999; Davis et al., 2002).

The use of steroids such as PA to study the effects of stress on fish immunity has been documented in several fish species (McCarthy, 1977; Hiney et al., 1994; Cipriano et al., 1997). It is important to note that stress responses in fish can vary from genus to genus, species to species and even from fish to fish (Mommsen et al., 1999; Barton, 2002). An individual fish response to stress was seen here in that the 30 catfish varied in their protein stress results to PA-injection when compared weekly with their own serum protein levels. The long-term effects in Ictalurid catfish from IP injection with PA on serum and innate immune protein concentrations have not before been reported. These findings could be beneficial to the aquaculture industry (Tables 4.1 and 4.2).

Catfish with highest pre-bled total protein concentrations were able to tolerate and survive injection with PA along with its induced long-term stress effects on protein

synthesis (Table 4.1 and Fig. 4.1). This indicates that a fish diet of substantial protein could be beneficial in catfish aquaculture to increase their pond survival. An increase in dietary protein may therefore lead to increased stress resistance along with increased disease resistance in pond aquaculture. Sham-injected control catfish showed no significant increase or decrease in total protein concentration, lysozyme or MBL levels when compared with their pre-bled sera levels (Figs. 4.1, 4.5, 4.6).

An important conclusion here was that catfish with the highest serum protein concentrations, including the innate immune proteins and globulins, should be better able to survive the many stressors of pond aquaculture. This can be seen in Group 1 catfish which had the highest pre-bled total protein concentration (mean 38.8 mg/ml) (Table 4.1). This would include the highest pre-bled concentrations of the innate immune proteins, lysozyme and MBL, as also seen in the Group 1 catfish (Table 4.2). The importance of an initial high protein concentration in catfish (Group 1) correlated very well ($P<0.001$) with their long-term survival and resistance to chronic suppression (Table 4.1).

Acknowledgements

The authors thank Dr. Brian Bosworth, Catfish Genetics Research Unit, U.S. Department of Agriculture, Stoneville, MS for providing the hybrid catfish. We thank Dr. Bill Simco, University of Memphis, for use of fish culture facilities at the Ecological Research Center. We also thank Dr. S. P. Wong, University of Memphis, for statistical analysis.

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Chapter 5: Conclusion

In Chapter 2, a C-type mannose-binding lectin was purified by affinity chromatography from sea lamprey (*Petromyzon marinus*) plasma (Figures 2.1 and 2.2). SDS-PAGE (12% gel) and Western blotting of the 2-ME reduced lamprey MBL (using guinea pig anti-MBL IgG as primary antibody) showed two molecular weight bands of 35 kDa and 65 kDa. This could indicate the presence of two different isoforms or subunits of the sea lamprey MBL. Only one N-terminal amino acid sequence was found, although two bands were seen by SDS-PAGE. This indicates that the N-terminal end amino acid sequence is the same for both molecular weight bands, but that the C-terminal end of the 35 kDa segment underwent post-translational processing. No band was seen to enter the gel with unreduced affinity-purified lamprey MBL by SDS-PAGE and Western blotting, indicating a molecular mass of large molecular weight.

Only one N-terminal end amino acid sequence was detected for the isolated lamprey MBL, which demonstrated purity of the affinity-purified MBL. No N-terminal blockage was found. N-terminal amino acid sequencing of the sea lamprey plasma MBL for the first 10 residues gave XXXTKGCPDA, where X represents an unidentified amino acid. A database Blast Search found that the sea lamprey plasma 4-10 amino acid sequence, as isolated here, matched with a 35 kDa subunit of C-type lectin isolated from sea lamprey (*P. marinus*) fish eggs for which a 16 amino acid sequence was obtained (Schluter et al. 1994). They did not determine if this C-type lectin was also found in sea lamprey plasma as was done in Chapter 2.

Sea lamprey plasma contained 261 μg of MBL/ml of plasma. The lamprey plasma protein concentration was 40.1 mg/ml. Lamprey MBL was therefore present in

lamprey plasma at 6.5 μg MBL/mg total protein. The presence of MBL in such high concentration in lamprey plasma could be important in innate immunity and their resistance to infection.

The initial mixing and loading buffers both contained calcium necessary for binding MBL to the mannan-affinity column. The C-type lectin binds mannose in a calcium-dependent manner as the lamprey lectin could subsequently be eluted from a mannan-agarose affinity column with 3 mM EDTA (Figure 2.1). EDTA is a chelator of calcium. The lamprey MBL can be considered then a C-type lectin (Ca-dependent) since it could be eluted from the mannan-agarose affinity column with EDTA. This also means that the lamprey C-type lectin has specificity for mannose. Lamprey MBL is likely a member then of the collectin family of Ca-dependent proteins.

The sea lamprey plasma MBL isolated in Chapter 2 had 14-times the concentration of MBL in μg/ml when compared with channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) serum MBLs. The sea lamprey plasma MBL must therefore play a very important role in lamprey innate immunity to infectious diseases when compared with the teleost bony fish.

In Chapter 2, a mannose-binding C-type lectin was purified from sea lamprey plasma by affinity chromatography (Figures 2.1 and 2. 2) and it specifically bound to mannose on the surface of *Aeromonas salmonicida* (Figure 2.3), a bacterial pathogen of the sea lamprey (Woo and Bruno 1999). Our initial finding in Chapter 2 of MBL in plasma of the sea lamprey (*P. marinus*) is important in understanding lamprey innate immunity and resistance to microbial pathogens. The presence of MBL in such high concentration in lamprey plasma could be important in their innate resistance to bacterial

infections, as the sea lamprey MBL binds to the pathogen *Aeromonas salmonicida* (Figure 2.3). Chapter 2 describes the presence of MBL in sea lamprey (*P. marinus*) plasma and evidence for a C-type lectin complement pathway of innate immunity.

The presence of lysozyme in fish serum was first reported by Lukyanenko (1965). The presence of lysozyme was previously reported in channel catfish serum and skin mucus at a concentration of 34 ng lysozyme/mg protein and 46 ng lysozyme/mg protein, respectively (Ourth, 1980; Ourth and Wilson, 1981). Lysozyme is most effective in hydrolysis of the peptidoglycan cell wall of Gram-positive bacteria. If the outer membrane of Gram-negative bacteria is damaged by innate immune factors or the complement system, then lysozyme can also hydrolyze the peptidoglycan cell wall layer of Gram-negative bacteria (Ryan and Ray, 2004). Fish lysozyme may have a greater activity spectrum when compared with mammalian lysozyme, since it can be active against both Gram-positive bacteria and Gram-negative bacteria (Watts et al., 2001). This may be due to the presence of different isoforms of fish lysozyme as found in rainbow trout (Grinde et al., 1988). The sea lamprey lysozyme isolated in Chapter 3 (Fig. 3.2) was only effective against Gram-positive bacteria (Table 3.1). This indicates perhaps a difference between sea lamprey lysozyme and the lysozymes of many teleost fish (Jolles and Jolles, 1984; Grinde et al., 1988).

The lamprey plasma and lysozyme were most effective against *M. luteus*. Lysozyme was also effective against *B. megaterium* and *M. phlei* (Table 3.1). Mycobacterial species are important pathogens of many teleost fish including striped bass of the Chesapeake Bay area USA. Mycobacteriosis is of concern to commercial

fishermen in the Chesapeake Bay, as one species of *Mycobacterium* is a zoonotic agent that can potentially infect humans (Ottinger et al., 2003).

Antifungal peptides, like the defensins, have been identified in insects and mammals (Andra et al., 2001; Zasloff, 2000; Sahl and Bierbaum, 2008). α-Defensins are found in granules of neutrophils and have antifungal and antibacterial activity. α-Defensins are pore-forming, low molecular weight peptides active against fungal cell membranes including *A. flavus* and *A. fumigatus* (DeLucca and Walsh, 1999 and 2000). Antifungal activity by the peptide Cecropin A has also been found against *Aspergillus* species (DeLucca et al., 1997). Fungal species are widely found in the environment including soil and decaying organic matter and can pose a disease threat to fish.

Antifungal plasma activity against *P. notatum* and *A. flavus* was seen (Table 3.1). Fraction #49 (Fig. 3.3) from P-10 gel filtration chromatography demonstrated antifungal activity. The antifungal clearing around the well, using 6 µg/well of sea lamprey antifungal peptide, was nearly equivalent to antifungal susceptibility testing when using 25 µg/well of known antifungal azole compounds (Magaldi et al., 2004).

The sea lamprey plasma was not effective against *E. coli* or *A. salmonicida* which indicates that lysozyme does not have lytic activity against Gram-negative bacteria. The eluted fractions from P-10 column chromatography also showed no bactericidal activity against *E. coli* or *A. salmonicida*. This indicates the absence of antibacterial peptides in both plasma and P-10 fractions (Chung and Ourth, 2000; Ourth and Chung, 2004). Yun and Li (2007) also did not find antibacterial peptides in sea lamprey leukocytes**.** No innate immunity to *A. salmonicida* was found, indicating that this bacterial species could

serve as a potential pathogen in the lamprey natural environment (Ourth et al., 2008). The lamprey MBL as isolated in Chapter 2 did however bind to *A. salmonicida*.

The molecular weight of the sea lamprey lysozyme isolated in Chapter 3 (14.3 kDa) was equivalent to hen egg white lysozyme (Fig. 3.2). This compares with 14.4 kDa for rainbow trout (*Oncorhynchus mykiss*) lysozyme (Grinde et al., 1988), 14.5 kDa for coho salmon (*Oncorhynchus kisutch*) lysozyme (Yousif et al., 1991), and 14.5 kDa for Atlantic salmon (*Salmo salar*) lysozyme (Fagan et al., 2003).

In Chapter 3, lysozyme was detected in the sea lamprey plasma pool at a concentration of 5 μg lysozyme/mg total protein. When compared with channel catfish lysozyme determined at the same time (222 ng lysozyme/mg total protein), the sea lamprey was 23-times greater in lysozyme concentration than the channel catfish lysozyme. The large difference in lysozyme concentration between sea lamprey and channel catfish could be because the channel catfish, an advanced teleost fish, has both innate immunity and adaptive immunity to rely on. In contrast, the sea lamprey which is the most primitive fish, has only innate immunity. The lamprey may therefore compensate by producing a much greater concentration of plasma lysozyme when compared with the channel catfish.

Squalamine, a non-peptide antimicrobial factor of innate immunity, has been found in white blood cells of the sea lamprey (Yun and Li, 2007). A squalamine-like compound (655 Daltons) was identified in channel catfish leukocytes and may play a role in innate immunity (Ourth and Chung, 2004). MBL, an important component of innate immunity (Kindt et al., 2007) was identified and characterized in this sea lamprey species in Chapter 2 and also in the channel catfish (Ourth et al., 2007).

Lysozyme and antifungal peptides are important components of innate immunity in animals (DeLucca and Walsh, 1999 and 2000; Kindt et al., 2007). The data indicate that the high concentration of both plasma lysozyme and antifungal peptide found in Chapter 3 could be very important in sea lamprey resistance to bacterial and fungal infections. Fungal infections must be important in lamprey health and innate immunity, since this sea lamprey species produces its own antifungal peptide.

In Chapter 4, stress induced by prednisolone acetate (PA) injection significantly decreased protein synthesis in the three groups of catfish. The ten catfish in Group 1 survived for all six weeks of the study. These ten catfish also had the highest concentrations of total protein before PA injection (Table 4.1). Group 1 catfish also had the highest pre-bled globulin, lysozyme and MBL concentrations (Tables 4.1 and 4.2). Group 2 catfish had the highest pre-bled albumin concentration and survived for four weeks, which includes three weeks post-injection with PA (Table 4.1).

The globulin concentrations in Table 4.1 may indicate the concentrations of IgM globulin for the three groups of catfish. By the end of the study, Group 1 globulin concentration, which contained the highest pre-bled level of globulin, had decreased four-times and this may indicate a decrease in fish IgM globulin (Table 4.1). This indicates that a long-term response to chemical stress could also negatively affect the adaptive immune response of catfish. Sahoo and Mukherjee (2001) demonstrated an immunosuppressive effect of chronic stress on total protein and globulin synthesis but not albumin synthesis in Indian major carp (*Labeo rohita*) after IP injection with aflatoxin B_1.

The initial increase one week after PA injection in the concentrations of the innate immune proteins lysozyme and MBL (Table 4.2) supports the concept that acute or short-

term stress can be beneficial in catfish immunity (Maule et al., 1989; Demers and Bayne, 1997; Bilodeau et al., 2003, 2005). However, two weeks after injection with PA, the concentrations of both lysozyme and MBL decreased significantly ($P<0.001$) in Groups 1 and 2 indicating that the long-term effects of stress could be detrimental to catfish innate immunity. The innate immune response is of prime importance in fish (Tort et al., 2003).

Bilodeau et al. (2005) found that lysozyme activity in channel catfish with susceptibility differences to *Edwardsiella ictaluri* remained elevated in both susceptible and resistant catfish families for 20 days following challenge with the bacterium. The only difference was the onset of lysozyme activity. The resistant family of channel catfish had elevated lysozyme activity beginning one day after challenge with *E. ictaluri*. The susceptible family did not have elevated lysozyme activity until day two. They did not find that their data supported a link between serum cortisol and lysozyme activity. However, Yin et al. (1995) found that chronic stress caused by over-crowding in fancy carp (*Cyprinus carpio*) led to an overall decrease in lysozyme activity. Lysozyme activity has been shown to increase and decrease depending on many variables, including toxicants and levels of stress (Saurabh and Sahoo, 2008). They found that lysozyme activity decreased with chronic stress. Lysozyme activity here initially increased one week after PA injection, but lysozyme activity dramatically decreased by the end of the week 6 (Table 4.2, Fig. 4.5). Lysozyme activity is therefore an important indicator of the innate immune response to stress in fish.

The occurrence of *E. ictaluri* infections in pond aquaculture of channel catfish can be stress-related and is well documented (Woo and Bruno, 1999; Bilodeau et al., 2003; Bilodeau et al., 2005; Small and Bilodeau, 2005). Stressors in ponds include stocking

densities of catfish, oxygen levels, ammonia, nitrite and temperature. Reducing the stress response of catfish can help in preventing enteric septicemia of catfish and other bacterial and microbial diseases (Woo and Bruno, 1999; Davis et al., 2002).

The use of steroids such as PA to study the effects of stress on fish immunity has been documented in several fish species (McCarthy, 1977; Hiney et al., 1994; Cipriano et al., 1997). It is important to note that stress responses in fish can vary from genus to genus, species to species and even from fish to fish (Mommsen et al., 1999; Barton, 2002). An individual fish response to stress was seen here in that the 30 catfish varied in their protein stress results to PA-injection when compared weekly with their own serum protein levels. The long-term effects in Ictalurid catfish from IP injection with PA on serum and innate immune protein concentrations have not before been reported. These findings could be beneficial to the aquaculture industry (Tables 4.1 and 4.2).

Catfish with highest pre-bled total protein concentrations were able to tolerate and survive injection with PA along with its induced long-term stress effects on protein synthesis (Table 4.1 and Fig. 4.1). This indicates that a fish diet of substantial protein would be beneficial in catfish aquaculture to increase their pond survival to stress. An increase in dietary protein could therefore lead to increased stress resistance along with increased disease resistance in pond aquaculture.

An important conclusion in Chapter 4 was that catfish with the highest serum protein concentrations, including innate immune proteins and globulins, should be better able to survive the many stressors of pond aquaculture. This can be seen in Group 1 catfish which had the highest pre-bled total protein concentration (mean 38.8 mg/ml) (Table 4.1). This would include the highest pre-bled concentrations of innate immune

proteins, lysozyme and MBL, as also seen in the Group 1 catfish (Table 4.2). The importance of an initial high protein concentration in catfish (Group 1) correlated very well ($P<0.001$) with their survival and resistance to long-term suppression (Table 4.1).

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